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FRANK R. LILLIE

1896 - 1915

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On the Smallest Parts of Stentor Capable of Regeneration

A CONTRIBUTION

ON THE

Limits of Divisibility of Living Matter

BY

FRANK R. LILLIE

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ON THE SMALLEST PARTS OF STENTOR CAPABLE OF REGENERATION; A CONTRIBUTION ON THE LIMITS OF DIVISIBILITY OF LIVING MATTER.

FRANK R. LILLIE.

In experiments on the power of multiple development of the ovum the result has been reached, that a portion of less volume than one-fourth that of the normal ovum does not possess the capacity of producing an *embryo* or *larva*, though it may a gastrula (see postscript); while a portion equal to one-fourth or more of the volume of the normal ovum may, under suitable conditions, produce a gastrula and finally a larva of corresponding relative bulk. This result has been attained by Loeb,[1] Wilson,[2] Driesch,[3] Morgan,[4] and Zoja.[5] Wilson found only a single larva of Amphioxus of one-fourth the normal size and that showed several defects; and Driesch has not, so far as I am aware, mentioned any pluteus of less than one-quarter size. Morgan has recently published the results of his studies on the power of multiple development of the echinoderm ovum. In this he shows that "the volume of the smallest gastrula which can be produced from fragments of the egg falls below $\frac{1}{64}$ the volume of normal gastrulae. The volume of the fragments of the egg which produced such gastrulae, varies between $\frac{1}{40}$ and $\frac{1}{50}$ of the volume of the ovum." (Summary p. 124 *loc. cit.*) But these smallest gastrulae were unable to

[1] Jacques Loeb. "On the Limits of Divisibility of Living Matter." In *Biol. Lectures of Marine Biol. Lab. for 1894.* Boston, Ginn & Co. Also in *Archiv für Ges. Physiologie von Pflüger.* Vol. LIX. 1894.

[2] E. B. Wilson. "Amphioxus and the Mosaic Theory of Development." JOURNAL OF MORPHOLOGY. Vol. VIII, No. 3. 1893.

[3] Driesch. "Entwicklungsmechanische Studien." III–VI. *Zeitschr. f. wiss. Zool.* Bd. LV, p. 9. 1893.

[4] "Studies of the Partial Larvae of Sphaerechinus," by T. H. Morgan, in Roux's *Archiv für Entwicklungsmechanik der Organismen.* Bd. II, H. 1. 1895.

[5] Sullo sviluppo dei blastomeri isolati dalle uova di alcune meduse (e di altri organismi) per il Dr. Raffaello Zoja; in Roux's *Archiv für Entwicklungsmechanik der Organismen.* Bd. II, H. 1. 1895.

develop further. Morgan himself says (p. 117): "The smallest pluteus which I have found measured 7×8, and the normal form in the same dish 12×15. Another larva at the beginning of the pluteus stage measured 6×7. The larvae have apparently one-eighth the volume of the normal, and correspond in size very nearly to the pluteus figured by Loeb. If, however, we compare these small larvae with the larvae derived from isolated blastomeres, the conclusion is forced upon one that these plutei have in all probability come from fragments of the egg having only about *one-half to one-fourth of the volume of the egg*." Inasmuch as the test proposed for the limits of divisibility rests upon the capacity for complete development to an *embryo*, or *larva* properly so-called, it is only these last figures of Morgan's that demand consideration. It would seem from these that Loeb (*loc. cit.*) has made his figure, *one-eighth*, too low, not having taken in account the fact, emphasized by Morgan, that the growth of the small blastulae, gastrulae and plutei is less rapid than that of the normal.

Zoja's results on the separation of the blastomeres of the ova of certain medusae must also be considered. In the summary, p. 32, we find the following remarks: "Medusae; Die Entwicklung der getrennten Blastomeren ($\frac{1}{2}$ und $\frac{1}{4}$ Ei von Liriope mucronata, Geryonia proboscidalis, und Mitrocoma annae; $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, Ei von Clytia flavidula und Laodice cruciata) ist ganz genau in allen ihren Phasen wie diejenige des ganzen Eies." — "Es bildet sich endlich immer eine schwimmende Larva, aus zwei Gewebe bestehend, die von jener, welche aus $\frac{1}{1}$ Ei hervorgeht, nicht unterscheidbar ist, ausser in den Dimensionen."

From this it appears that a $\frac{1}{16}$ blastomere of Clytia and Laodice is able to develop into a swimming larva. But from the next statement I judge that the development cannot go farther; this is: "Bei Clytia zeigten $\frac{1}{2}$ und $\frac{1}{4}$ Ei auch die vollständig entwickelte idroide Form, und bei Liriope gab $\frac{1}{2}$ eine kleine runde Medusa, in welcher die vier primären Tentaculi normalerweise im Kreuz angeordnet waren." Thus a fourth blastomere is the smallest portion capable of complete development.

There are three possible explanations of this failure of such small parts to develop:

1. That the whole organization of the species cannot be included in so small a space. Briefly, *deficient organization.*

2. That so small a volume of matter cannot fulfill the mechanical conditions consequent on cell-division, formation of a segmentation cavity, invagination, and so forth, owing perhaps to increased surface tension (Driesch), not to mention other conceivable alterations of the extrinsic factors of development.

3. That such a small part "is not able to set free that amount of energy which would be required for its transformation into a gastrula or a pluteus." (Loeb.)[1]

The third explanation seems to me inadequate; because such masses may continue to live for a considerable period of time and display an amount of energy in *atypical* form changes and rapid ciliary movement, which would suffice to produce the phenomena of normal development, did not other factors (included in the first or second of the above alternatives) prevent. Moreover, it is well known that exceedingly minute protoplasmic bodies, very much smaller than one-eighth the echinoderm ovum, may produce a relatively enormous amount of energy: *e.g.* bacteria and spermatozoa. Finally we do not know how much of developmental energy is of intrinsic and how much of extrinsic origin. *We are limited, then, to the first two alternatives.*

Now, in the regeneration of a unicellular organism those mechanical conditions consequent on cell-division, formation of a segmentation cavity, invagination, and so forth, are not required to be fulfilled. Surface tension and other extrinsic factors of development of Metazoa have not been shown to exercise a controlling influence in the regeneration of such an organism.[2] It occured to me, therefore, that the *ciliate Infusoria*

[1] Morgan's explanation, that the failure to develop is due to inability to produce a sufficient number of cells for the next ontagenetic stage, will come under the first or second of these alternatives, according to the general point of view.

[2] Of course it is possible for any one to maintain that extrinsic forces do control the regeneration. But the burden of proof rests upon the maker of such an assumption.

offer conditions for decision between these two alternatives. If it should be shown that a nucleated portion of the body below a certain minimal size is incapable of regeneration, the first alternative would receive support. If, on the other hand, the smallest nucleated fragments of the body are capable of regeneration with restoration of the normal form, the first hypothesis would fall, and the second tend to be established.

My material consisted of two species of Stentor, viz.: *S. polymorphus* and *S. coeruleus*. The former species occurred in immense profusion on decaying leaves of the water-lily in a small pond near Ann Arbor, the latter appeared in considerable numbers in a small aquarium which had stood in the laboratory for six or seven weeks, and contained gatherings from a swamp. This species is more favorable for experimental work than the former, because the protoplasm is transparent, enabling one to see the nucleus readily in the living animal. In *S. polymorphus* the body is rendered almost perfectly opaque by the presence of immense numbers of symbiotic unicellular Algae, the so-called zoochlorellae, which either hide the nucleus completely from view, or permit mere momentary glimpses of it. On account of the ease of procuring any desired supply of *S. polymorphus* my work was done chiefly on this form; but the results were checked on *S. coeruleus*, and were practically the same for both species.

To reach the desired result it was necessary to find or devise a method by which nucleated fragments of every possible size, beginning with a portion not much larger than a single node of the nucleus, could be produced in large numbers; for reliable quantitative results can be reached only by observation of a large number of cases of regeneration. For this purpose I tried the method of *shaking* which has yielded such admirable results with the animal ovum in the hands of Wilson, Driesch, Morgan, and others, and found it to succeed to perfection. If a number of Stentors are put in a small vial about one-third filled with water and shaken quite violently from five to twenty times (*S. coeruleus* requires to be shaken only about five times; *S. polymorphus* ten to twenty times), and then examined under a low power of the microscope, one sees that

the animals have been broken into numerous fragments of every possible size and shape. In the field of the microscope there are present at the same time naked nodes of the nucleus either single or united in groups of two or three, and parts of the body, both nucleated and unnucleated, ranging in size through every possible gradation from 25μ in diameter to about 200μ. Most of the latter are being driven hither and thither by the action of the cilia with which they are covered; and many of them are of the most bizarre and curious shapes: T-shaped, Y-shaped, or provided with other arm-like processes, or of forms impossible to describe; but most of them are of more regular form, triangular, quadrilateral, oval, and spherical.

The moniliform character of the nucleus in these species of Stentor insures that a large proportion even of the smallest pieces receive at least some part of the nucleus. In order to satisfy myself that such is the case, I killed and stained the whole of one lot of *S. polymorphus*, which had been shaken as described, about fifteen minutes after the operation. The stained material was then mounted in balsam and measurements were made of the smallest nucleated pieces. Some of the measurements were as follows:

1. Naked nodes of nucleus, spherical or oval; 20–25μ.

2. A spherical piece 31μ in diameter containing a single node of the nucleus. Nucleus excentric. Protoplasm a thin cortex.

3. A spherical piece 37μ in diameter; contained a single node of the nucleus.

4. A spherical piece 37μ in diameter; contained two nodes of the nucleus.

5. A spherical piece 40μ in diameter; contained six nodes of the nucleus.

6. A spherical piece 50μ in diameter; contained one node of the nucleus.

7. A spherical piece 50μ in diameter; contained two nodes of the nucleus.

8. A spherical piece 50μ in diameter; contained four nodes of the nucleus.

9. A spherical piece 66μ in diameter; contained a single elongated node of the nucleus.

10. A spherical piece 69μ in diameter; contained seven nodes of the nucleus.

Other similar pieces containing one or more nodes of the nucleus were seen; some were of course not spherical, but I have given the spherical pieces as easier to compute the volume. Of larger nucleated pieces there was no lack; they were very numerous, as one would expect. There were, of course, numerous unnucleated masses of protoplasm of various sizes, but few large pieces. In fact, the majority of the pieces below 100μ in diameter were unnucleated; but the above list shows that a good many of such small pieces contained one or more nodes of the nucleus. I did not attempt to ascertain what was the proportion of nucleated to unnucleated pieces of such small size; but it must have been quite large, perhaps one to ten or even more.

One further remark as to my methods. When any doubt existed in my mind as to the presence of parts of the nucleus in examples noted, the specimen was killed and stained, generally in Schneider's aceto-carmine, and the actual condition of the nucleus thus determined with certainty. This of course involved the sacrifice of a great deal of material.

In consequence of the often curious and asymmetrical shapes of the pieces produced by shaking, I expected to obtain valuable results on the teratogeny of the Stentors for comparison with the results of Balbiani and Johnson, but I have been almost completely disappointed in this respect. When regeneration of a piece takes place at all, it almost always happens that a single more or less perfect animal of typical form results.

RESULTS.

In this paper I shall speak only of results obtained on the smallest parts capable of regeneration, leaving other questions suggested by the experiments for future consideration. From numerous experiments, involving many hundreds of *S. polymorphus*, it was found that the smallest parts capable of

regeneration possess the volume of a sphere of about 80μ diameter. A lesser number of experiments on a smaller number of *S. coeruleus* yielded results almost identical. In the following list I give measurements of some of the smallest Stentors found. After measuring in a more or less expanded condition the animals were made to contract, when they assumed almost the form of a sphere; the diameter of the sphere was then measured, and this measurement was used for comparison.

1. *Stentor coeruleus.* 45½ hours after shaking. Regeneration was complete or nearly so. I could see the adoral spiral sinking into the oesophagus, the mouth, and contractile vacuole. When expanded the form was quite typical. There were two *separated nodes* of the nucleus present.

Measurements. None were made of the expanded animal. Diameter of contracted animal (spherical) 90μ.

2. *S. polymorphus.* 67 hours after shaking. Regeneration complete.

Measurements. a. Expanded, 257μ in length; 80μ across frontal field. *b.* Diameter of contracted animal (spherical) 80μ.

3. *S. polymorphus.* 70 hours after shaking. Regeneration complete.

Measurements. a. Expanded, 257μ in length; 84μ across frontal field. *b.* Diameter of contracted specimen (spherical) 87μ.

4. *S. polymorphus.* 96 hours after shaking. Regeneration complete. The animal was sluggish and did not expand well.

Measurement. Diameter of contracted specimen 75μ.[1]

I have measurements of a number of Stentors of slightly larger size than the ones given; but the smallest Stentors were very scarce. By far the greater number of nucleated parts, which possessed a spherical diameter of less than 100μ, were incapable of regeneration, or at any rate did not regenerate. However, but a single example is sufficient to show that a portion of the volume of the example in question is capable of regeneration.

[1] My note-book expresses a little doubt about this specimen, but it was certainly under 80μ.

The volume of the smallest Stentor found was thus equal to a sphere of somewhat less than 80μ in diameter. Not one of the hundreds of smaller nucleated parts regenerated, though I found one part, 71μ in diameter in spherical form, which had assumed a fairly typical form of semi-contraction and possessed a single bead of the nucleus; anterior and posterior ends (or foot) were thus recognizable, but there was neither oesophagus nor adoral membranellae present. Even if we admit this as regenerated, which I do not, it does not essentially alter the final result.

My conclusion is, therefore, that nucleated parts of *Stentor polymorphus* of less volume than a sphere of 80μ (approximately) in diameter are incapable of regeneration; nucleated parts of greater volume are capable under favorable conditions of complete regeneration.

The main results hitherto reached on the merotomy of the Protozoa can be summarized as follows:

1. Cytoplasm without nucleus is incapable of regeneration (Nussbaum, Gruber, Verworn, Balbiani, and others). This I can confirm. (Verworn has shown that the isolated central capsule of *Thalassicola nucleata* from which the nucleus has been removed is capable of partial regeneration, but it soon goes to pieces. Gruber has shown that if a Stentor in process of fission be transversely divided so that the posterior part receives no nucleus, this part is nevertheless able to regenerate.)

2. Nucleus without cytoplasm is incapable of regeneration. (Verworn, Balbiani.) This also I can confirm.

3. Portions of the body consisting of nucleus and cytoplasm are capable of regeneration. *To this I must add: provided that the amount of cytoplasm exceed a certain minimal volume* (which in the case of Stentor at any rate is quite considerable).

This amounts to a demonstration of Verworn's view that regeneration in the Protozoa is due to the reciprocal interaction of nucleus and cytoplasm. Organization resides in the cytoplasm as well as in the nucleus. How otherwise are we to explain the fact that a difference in the amount of cytoplasm alone (equivalent to the difference in volume of two spheres of 80 and 70

micromillimeters respectively) determines the occurrence of regeneration?

As regards the bearing of the results on the limits of divisibility of living matter: we are not concerned here with the question of the ultimate constitution of protoplasm, its composition of any ultimate vital elements whatsoever, but merely with the question propounded by Loeb, "What is the order of magnitude of the smallest particle that can show all the phenomena of life?" In the case of the animal ovum as already noted this is about one-fourth of its volume, if we include development as one of the phenomena of life. Certainly development includes all the phenomena of life. In the case of Stentor the volume is relatively considerably less as the following calculation will show:

The volume of the smallest perfect *Stentor polymorphus*, which I was able to produce, was equal to that of a sphere of about 80μ diameter; the average volume of the Stentors used in the experiments was equal to that of a sphere of about 230μ, as I determined from a series of measurements of animals killed in a weak killing-fluid, and thus completely contracted. That is, the ratio of the diameters of the smallest and the average Stentor is about 1:3; or the ratio of volume to volume is about 1:27. That is to say that the smallest Stentor which can be produced is about one twenty-seventh of the volume of the average Stentor.[1] This number is of course a mere approximation, but it certainly will not be made any greater by subsequent investigation, though it may be lessened somewhat.

[1] In the case of *S. coeruleus* the figures are different: the smallest measurement which I have of this species is 90μ; the average is 280μ; thus the ratio of the smallest to the average is about 1:3 in terms of the diameter, or 1:27 in terms of volume. I believe, however, that it would be possible to produce a smaller *S. coeruleus* by working over a larger amount of material. I do not think that there is much difference in the *absolute* size of the smallest Stentors which can be produced, whether one uses the largest or smallest normal specimens. If *e.g.* the average size of a lot of large Stentors were 320μ, the smallest specimen which could be produced would still be 80μ. The ratio of volumes would then be 1:64. Of course this does not necessarily mean that 64 Stentors could theoretically be produced at one time from a single one, for I doubt that the nucleus could undergo that amount of division.

In any case this relation forms a striking contrast to that found in the development of the animal ovum, where a portion of less volume than one-fourth that of the ovum does not develop into an embryo (see postscript). It has been very generally found that a portion (of the two- or four-cell stage) equal to one-eighth the volume of the ovum never develops farther than the gastrula stage.

In the case of the animal ovum, again, parts slightly smaller than the minimum necessary for the complete development may undergo partial development. In Stentor we have a parallel phenomenon: parts of less than 80μ spherical diameter may undergo partial regeneration, but are unable to complete it.

It seems to me that neither increased surface tension due to diminution of surface area, nor yet any other external factor, is responsible for this failure of small pieces of Stentor to regenerate. The cause lies within; and I do not believe that it is to be sought in an insufficient production of energy. For such small pieces may live for days, constantly producing and expending energy in the ordinary processes of metabolism. I am forced, therefore, to the conclusion that the organization of these parts is in some way deficient. *There is probably for each species of animals a minimal mass of definite size consisting of nucleus and cytoplasm within which the organization of the species can just find its latent expression.* This is the *minimal organization mass.*

In the case of the Protozoa the size of this minimal mass is that of the smallest part capable of complete regeneration. But I do not believe that in the Metazoa the minimal organization mass is that of the smallest part of the ovum capable of developing into a normal embryo; for undoubtedly the influence of external factors is of the greatest importance here. I would conceive then that in the Metazoa this hypothetical minimal organization mass is smaller than any part yet observed to develop into a normal embryo. Still, from my results on Stentor, I believe that it is of such a size as to be easily visible under a low power of the microscope.

ZOÖLOGICAL LABORATORY OF THE
UNIVERSITY OF MICHIGAN, Dec., 1895.

POSTSCRIPT. — After sending the above article to the editor I had access to Boveri's recent paper entitled "Ueber die Befruchtungs- und Entwickelungsfähigkeit kernloser Seeigeleier und über die Möglichkeit ihrer Bastardirung," published in Bd. II, Heft 3, of Roux's *Archiv für Entwickelungsmechanik der Organismen*, Oct. 22, 1895. Boveri states that the smallest dwarf larva which he obtained came from a fragment which could not have measured more than $\frac{1}{20}$ the volume of the intact ovum "bei ungünstiger Rechnung." His conclusion is: "Das Fragment des Seeigeleies bis herab zu einer Grösse von $\frac{1}{20}$ des ursprünglichen Eivolumens besitzt die formative Wertigkeit des ganzen Eies." This is in marked contrast to the results of the other authors quoted, none of whom have found a figure less than $\frac{1}{4}$. The difference may be due in part to the fact that Boveri shook the ova before fertilization, while the other experimenters performed this or an analogous operation after fertilization; although this does not seem very probable. If the exact proportion of the minimal organization mass to the whole ovum be a matter of any importance, very great care in the estimation of the volumes of dwarf larvae would seem to be necessary, taking into account the differences in thickness of the layers in dwarf and normal larvae, and also the relatively slow increase in volume of the former.

The figure which I have found for Stentor is but little lower than that of Boveri for the animal ovum, and this approximation suggests interesting comparisons.

THIRD LECTURE.

ADAPTATION IN CLEAVAGE.

FRANK R. LILLIE.

I. Introduction.

It has happened, very naturally, that writers on the subject of cell-lineage have laid special emphasis on the resemblances, which are nothing short of marvellous from the older points of view, between the cleavage of the eggs of even widely separated forms. Over and over again it has been demonstrated that in gasteropods, lamellibranchs, annelids, and turbellarians, the ectoderm is made up of three quartets of cells, formed from the first four cells; that the fourth product of the left posterior macromere contains the mesoblast, excepting in the turbellarians; that the turbellarian mesoblast is represented in the other groups; that the four basal cells, after the separation of the ectoderm and mesoblast, form endoderm; that the ectoderm of the entire trunk is derived from d^2 (speaking technically), and that there is a wide-reaching sameness in the cell-lineage of the prototroch, cross, and of other larval organs. Ancestral reminiscences even have been discovered in the cleavage (E. B. Wilson, 14). I do not in the least underestimate the immense value of these results, which form one of the most brilliant and interesting chapters in modern embryology. But the tendency to schematize has naturally arisen, and one of the most instructive aspects of cell-lineage is thus lost sight of, namely, *the special features of the cleavage in each species, which are, I believe, as definitely adapted to the needs of the future larva as is the latter to the actual conditions of its environment.*

This principle is meant to apply only to the "determinate type of cleavage" (Conklin), in which larval, and hence adult structures, can be shown to have a definite cell-lineage extending back to the unsegmented ovum. The illustrations and arguments will be drawn, first, from the cleavage of the egg of a fresh-water lamellibranch, *Unio*, which I have specially studied, and then, to secure a broader foundation in fact, some significant variations in the cleavage of the eggs of annelids will be considered. This theory was first used, I believe, for the explanation of determinate cleavage in my papers on the "Embryology of the *Unionidæ*" (6 and 7), where I pointed out that the size of the cells and the rate and direction of their cleavages "are ruled by the needs of the embryo," and concluded: "The peculiarities of the cleavage in *Unio* are but a reflection of the structure of the glochidium, the organization of which controls and moulds the nascent material." In his lecture on "Cleavage and Differentiation" (*Biological Lectures*, 1896–97), Conklin has maintained a similar view, *viz.*, "that all differential cleavages . . . are directly and causally related to the uses to which these cells are put." In the second part of this paper the retrospective significance of this principle will be considered in the light of some observations on the maturation, fertilization, and first two cleavages of the egg of *Unio*.

If we are to understand the special features of the cleavage in *Unio*, which are, I believe, purely adaptive, it will be necessary, first of all, to describe the peculiar larva. The glochidium (Fig. 1) possesses two shell-valves, shaped something like the bowls of two spoons cut across near their bases, and united by a central hinge. The curvature of the anterior margin of each valve is somewhat greater than that of the posterior margin, and each lateral angle is provided with a strong hinged hook, which is secondarily toothed in *Anodonta* on its outer face. The valves may be closed with great force by a single strong adductor muscle, the main bulk of which is somewhat anterior to the middle of the body. The interior of the spoon-shaped valves is lined by the larval mantle, composed of very large five or six sided cells, in the exact centre of which is the opening

of a long tubular gland, the thread-gland, coiled several times round in the right valve of the shell. The secretion of this gland takes the form of a long thread, which can, apparently, be respun when lost, and which floats freely in the water. There are four tufts of sense-hairs on each side of the larval mantle, three arranged in the form of a right-angled triangle just within the outer angle of each valve, and one on either side of the middle line, a trifle behind the opening of the thread-gland.

Now all of the structures mentioned thus far, the shell, the larval mantle, the adductor muscle, the thread-gland, and the

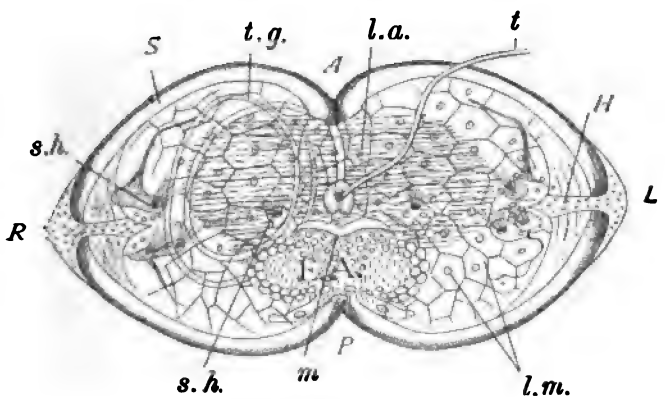

FIG. 1.—Glochidium of *Anodonta*, one of the *Unionidæ*, lying on its dorsal surface, with the shells expanded so as to show the soft parts. *A*, anterior; *P*, posterior; *R*, right; *L*, left; *S*, shell; *H*, hook; *l. m.*, larval mantle; *l. a.*, larval adductor muscle; *t. g.*, thread-gland; *t.*, thread; *s.h.*, sensory hairs; *m.*, mouth; *E. A.*, embryonic area.

tufts of sense-hairs, are purely larval organs, destined to form no part of the adult, but to degenerate. They are already old when the life of the individual has hardly begun, and they form by far the greater bulk of the larval body. The embryonic material, from which the body of the adult is to develop, is all included within a small area (*E. A.*) just in front of the posterior angle of the valves of the shell. This area is only about one-third of the length of the glochidium, and less than a third of its width, when the valves are expanded; yet within it we find the rudiments of the intestine with minute liver-diverticula and end-gut well marked but no opening to the exterior, of the mesoblastic structures, and of the foot, mantle, and gills.

All these parts are entirely functionless, and great only in potentialities.

The glochidium is a larva highly specialized for a peculiar mode of development. When the larva is extruded into the water by the mother-clam, it falls to the bottom and lies there with its thread floating in the water, violently snapping its valves together at the least disturbance. It is really a most sensitive little creature, and so readily perceives any fish swimming by. If it be so fortunate as to have its thread caught on the fish, it at once attacks it with its hooks. It is, of course, only on the unprotected parts of the fish, such as the fins or the gills, that the larva is able to make any impression; and it is stated that the larvæ of *Anodonta* usually become parasitic on the fins, and those of *Unio* on the gills. The irritation caused by the presence of the larva sets up a proliferation of the epidermis, which speedily encloses the little parasite in a sort of cyst, within which the later development takes place.

II. Cleavage of the Egg of Unio.

Adaptation in cleavage can manifest itself only in the three possible modes of cleavage variation, which are, as has been pointed out by Mead, Jennings, zur Strassen, and others, first, differences in the rate of cleavage of cells; second, differences in size; and third, differences in the direction of the cleavage or position of the resulting parts. Let us now examine the cleavage of the egg of *Unio* with these general principles and the structure of the larva in mind. I may as well state at once that I regard the *general form* of the cleavage as inherited from a long series of ancestors, extending back, probably, to the *Turbellaria*.

The general plan of the cleavage is the same as in annelids, *Turbellaria*, and most mollusks, described in other lectures contained in this volume. From the first four cells, separated by meridional planes, the ectoderm is separated by three slightly oblique cleavages horizontal in their general direction, the first being dexiotropic, the second læotropic, and the third dexiotropic again. A fourth division of the left posterior macromere (basal cell) forms the mesoblast cell. (See Figs. 2–5.)

The first cleavage is meridional and very unequal, although the distribution of the yolk throughout the cytoplasm is perfectly uniform in the form of small granules (Fig. 20). The larger cell marks the position of the posterior end of the embryo. This cell divides before the smaller one, and unequally, the larger product again being posterior in position. The smaller cell of the two-celled stage then divides somewhat unequally, so that its larger product lies on the left side of the embryo. Thus we reach a four-celled stage (Fig. 2) composed of one very large cell, *D*, posterior in position, and three smaller cells, of which the left one, *A*, is the largest. The anterior and posterior cells, *B* and *D*, meet in a broad cross-furrow at the animal pole (Fig. 3), the cross-furrow at the vegetative pole being much smaller, contrary to the general rule in eggs of this type of cleavage. Thus the first two cleavages illustrate the three possible modes of variation, *viz.*, inequality of the cleavage products, difference in the rate of cleavage, and difference in the position of the cells, indicated by the broad cross-furrow at the animal pole. What is the meaning of these differences?

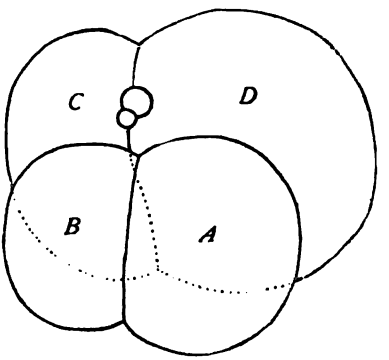

FIG. 2. — Four-celled stage of *Unio complanata* seen obliquely from the left side and above.

a. Relative Rates of Cleavage of the Constituent Cells. — Let us place side by side a table of the constitution of the thirty-two-celled stage of the egg of *Unio* and of an ideal ovum following, in direction of cleavage, the ordinary spiral type, with equal cleavage throughout, and the same rate of division in all the cells.

	IDEAL OVUM.	UNIO.
First generation of ectomeres	16	10
Second generation of ectomeres	8	13
Third generation of ectomeres	4	4
Fourth division of *D* (mesoblast cell)		1
Entomeres	4	4
	32	32

It is plain that as regards the relative rate of cleavage of the constituent cells, the egg of *Unio* departs very far from the ideal condition. Let us attempt to analyze this and see if these variations in the rate of cleavage are adaptive or not. The first thing we notice is that the number of the first generation of ectomeres is ten instead of sixteen; this means that these cells have divided more slowly than the average rate. Now in most mollusks and annelids they form the prototroch and the entire region in front of it, with the apical plate in the centre. But in *Unio* this region is degenerate, and I have never been able to find any trace of prototroch or apical sense-organ. It is plain, then, that the slow rate of cleavage is anticipatory of this, and is, in this sense, an adaptive modification. But why ten cells in place of eight? This means that two of the cells, c^1 and d^1, of this group have divided once oftener than their fellows. These cells occupy the posterior portion of the upper hemisphere, and later enter into the formation of the head-vesicle, and I believe that their more rapid cleavage is an early indication of their subsequent fate.

In the second generation of ectomeres we have a very different set of conditions as to number; the ideal number of cells of this generation in the thirty-two-celled stage is eight, but in *Unio* thirteen. This increase above the ideal number is due almost entirely to the rapidly succeeding divisions of one cell of this generation, d^2, but in part also to another, a^2. The other two cells, the anterior and the right members of the quartet, fall in with the ideal scheme, each having divided once. In this stage d^2 has broken up into six cells, a^2 into three. Is there anything adaptive in this hastening of the process of cleavage? d^2 is the cell from which the entire ectoderm of the trunk region is formed. The most characteristic thing about this region in the embryo of *Unio* is the relatively enormous size and very early formation of the shell-gland, and we have seen that the shell of the glochidium is of very special importance to it, not only as an organ of defence, but also of offence. Is it not wonderful that this important part should be represented, not only by the largest, but also by the most rapidly segmenting cell in the embryo, as though the egg knew

what was required of it, and acted accordingly? Can we deny that the rate of cleavage of this cell is an adaptive feature of the cleavage? The other cell of the second generation that divides more rapidly than the average rate is $a^{2\cdot2}$, or *Y*, the lower product of the first division of a^2. In the thirty-two-celled stage (Fig. 5) this cell already projects some distance into the segmentation cavity; after budding off two more small cells at the surface, it withdraws entirely within the segmentation cavity, and divides equally into two cells, which arrange themselves symmetrically on either side of the middle line. From these cells of the *larval mesoblast* the adductor muscle of the glochidium and certain isolated myocytes of the cleavage cavity are derived. Now the adductor muscle is an extremely large organ, of the utmost functional importance to the larva, and the variation in the rate of cleavage of the cells which are to form it appears to be adaptive.

The third generation of ectomeres possesses the normal number of cells for this stage, but it is interesting to notice that the posterior member, $d3$, is formed much before the others, owing to the tendency to more rapid division of the basal cell from which it arises. This cell, *D*, has also undergone another division, in excess of the divisions of the other basal cells, giving rise to the mesoderm proteloblast, $d4$ or *M*.

To summarize: groups of cells and even single cells vary greatly in their rates of segmentation. Each acts as though animated by an independent force. Moreover, these differences possess prospective significance, looking forward to the final outcome; and this phenomenon may fairly be called *adaptation in the rate of cleavage.*

But the objection may be raised: granted that the rate of division varies in these cells, and that those segmenting more rapidly form the earliest functioning parts, is it not possible that the variation is induced from without, and that this determines the subsequent fate? So far as we know, the only external factors which influence the rate of cleavage are temperature, chemical constitution of the medium, and perhaps some other general conditions of the environment, which must act uniformly on the whole egg, and hence cannot be held

accountable for differences in the rate of cleavage of constituent elements of the same egg. In addition to these factors, the presence of a larger proportion of yolk in certain cells may determine a slower rate of cleavage. But in the egg of *Unio* the yolk is distributed with perfect uniformity among the cells, so that each possesses practically the same relative quantity. Is it not, then, the absolute amount of yolk that determines the rate? No, unless one is willing to adopt the hypothesis that yolk tends to hasten the cleavage, for it is the largest cell in the egg that segments most rapidly. However, there is no constant relation between size and rate of cleavage, so even such an inverted hypothesis will not avail. There is, in fact, no escape from the conclusion that the factors determining differences in the rate of cleavage of the separate cells or definite cell-groups are of the same nature as those that determine differences between ova of different species, *viz.*, purely constitutional causes. In a very real sense, each cell in the segmenting egg of *Unio* acts, in respect to rate of cleavage, like an *ovum in ovo*.[1]

b. Variations in Size. — Although difference in size of daughter-cells in normal cleavage generally indicates difference in quality or constitution, it is not always safe to conclude that daughter-cells of the same size necessarily possess the same constitution. In other words, a division may be differential, even though the resulting cells are of the same bulk. The adaptiveness of determinate cleavage is illustrated in a manner even more striking than in differences of rate, by the inequality of certain divisions. I believe that it can be shown that the relative sizes of the cells in the early cleavages of the egg of *Unio* is adapted to the size and time of development of the larval organs. The first two cleavages are unequal with the result that two cells of the four-celled stage are larger than the two other equal ones. The largest of all contains the material of the two somatoblasts, and the next largest the material of the larval mesoblast.

[1] I believe that the entire egg is an undivided organism, and not a heap of equivalent cells, each of which may occupy any position in the whole, as Hertwig and Driesch have maintained. The above statement must be understood in this sense.

Let us now look at the first four divisions of *D*, the largest cell of the four-celled stage (Fig. 2). Its first division (Fig. 3)

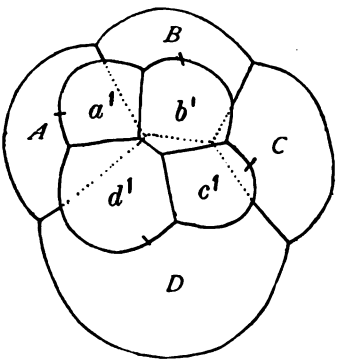

FIG. 3.

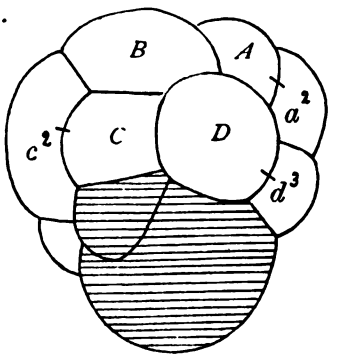

FIG. 4.

FIG. 3. — Eight-celled stage of *Unio complanata* from the animal pole.

FIG. 4. — Eighteen-celled stage of *Unio complanata* from the vegetative pole. The two cells ruled with horizontal lines are products, the first somatoblast, $d^2 = X$, which forms the trunk, including the shell-gland and foot of the larva.

is very unequal, and the smaller product, perhaps not one-tenth of the whole, lies nearer the animal pole; it is one of the first generation of ectomeres. The second division is likewise unequal (Fig. 4), but here the relations are reversed, for two-thirds, at least, of the substance of the cell passes into the upper product, the first somatoblast, d^2 or X; the third division is likewise unequal (D and $d3$, Fig. 4), and this time again the smaller product is uppermost, forming one of the relatively unimportant third generation of ectomeres. The fourth division, finally, is extremely unequal (Fig. 5), only a minute portion of the cell remaining at the lower pole, while the remainder forms the second somatoblast, d^4 or M, the proteloblast of the mesoderm. In each of these unequal divisions there is a manifest adaptation, the

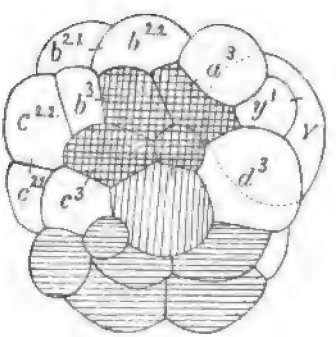

FIG. 5.—Thirty-two-celled stage of *Unio complanata* from the vegetative pole. The separation of the germ-layers is practically complete in this stage. Products of first somatoblast, $d^2 = X$, with horizontal lines; second somatoblast, $d^4 = M$ (mesoblast cell), with vertical lines; endoderm cells with both vertical and horizontal lines. $Y = a^{2\cdot2}$ larval mesoblast.

great bulk of the material passing into the two somatoblasts. In the thirty-two-celled stage (Fig. 5) the largest cells are those of the second generation of ectomeres, and this finds a sufficient explanation in the fact that the material of the first somatoblast, of the larval mesoblast, and of the larval mantle, is found in these cells. The relatively small size of the first and third generations of ectomeres and of the endoderm-cells is correlated with the relatively small part that they play in the upbuilding of the larva. The mesoderm proteloblast $d4$ is actually smaller than the larval mesoblast Y (Fig. 5) because of its deferred functional activity.

If there were any other explanation of these differences in size of the cleavage products than this seemingly teleological one, it might seem to simplify the problem of development. However, all theories that seek to explain inequalities of cleavage by the presence of yolk or the action of any known external force seem inadequate to explain the unequal cleavages in the egg of *Unio*.

c. Variations in the Direction of the Cleavage-Planes. — The early divisions follow the law of alternating spiral cleavage (Kofoid) for some time; and I shall not stop to consider these, although we are far from an adequate mechanical explanation of this law. I shall take up, instead, certain special divisions, which illustrate in a more striking manner the adaptiveness of variations in the direction of cleavage. The first somatoblast is established in the nine-celled stage by the second division of D; it is much the largest cell in the embryo, as we have seen, and it goes through with an exceedingly characteristic series of cleavages. It first buds off a small cell, x^1, on the right side of the vegetative pole, then another small cell, x^2, symmetrically placed on the left side, then a third small cell, $x3$, in the middle line, but towards the animal pole, then a fourth small cell, $x4$, in the middle line towards the vegetative pole (see Fig. 5); it then divides equally and bilaterally, and each half buds forth another small cell, $x5$, behind $x4$ on the vegetative pole. I do not know the fate of the cell $x3$, budded forth towards the animal pole. However, the other small products form the rudiment of the foot, which is an exceedingly small organ in the

larva ; and the large cells, which henceforward divide equally, the immense shell-gland. Can one imagine a more manifest adaptation? The small cells are placed just where they are needed, and are of the proper dimensions. Is there any other reason why the somatoblast should not have divided equally from the first, and the cells lying in one position have gone into the foot, and the rest into the shell-gland? If this were to happen, there would be no indication of adaptiveness in the cleavage, for then it might seem possible that, simply because of their different positions and conditions, part of the cells formed the foot and part the shell-gland.

d. To summarize.— In the cleavage of the egg of *Unio* there are marked variations in the size of the cells and in the rate and direction of their cleavages ; in every case, these possess prospective significance, and by means of them the organism is able, so to speak, to realize, in the most direct manner possible, on its available capital, the substance of the egg. *To this principle I have given the name of adaptation in cleavage.*

III. Adaptation in Cleavage in the Annelids.

In the annelids, as in the mollusks, the entire ectoblast of the trunk is segregated in a single cell, d^2 or *X*, known as the first somatoblast, and the mesoblastic germ-bands in the second somatoblast, d^4 or *M*. Now the cell-lineage of a great many annelids is known through the studies of Whitman (11), Wilson (13 and 14), Mead (9), Eisig (5), Treadwell (10), Child (1), and others. It is possible to arrange these annelids in a series according to the relative size of their somatoblasts. First there are forms like *Polygordius*, *Lepidonotus*, *Podarke*, *Hydroides*, and *Eupomatus*, with equal cleavage, in which the somatoblasts do not differ in size from the other cells of the same quartets (Fig. 6). Then in the order of increasing relative size of the somatoblasts come *Amphitrite* (Fig. 7) and *Chætopterus*, *Arenicola*, *Nereis limbata* (Fig. 8), *Clymenella* (Fig. 9), *Capitella*, *Aricia* (Fig. 10), *Scolecolepis* and *Spio*, *Nereis dumerilii*, and finally, *Clepsine* (Fig. 11). Now all of the forms with equal cleavage, although most widely separated in relationships, possess trocho-

phores characterized by an almost equatorial prototroch, a very large exumbrellar region, and relatively extremely slow development of the trunk. I merely allude to this here, because in another lecture of this series the causes of equal cleavage in the annelids are considered in detail by Mr. Treadwell. At the other end of the series comes *Clepsine* with its huge somatoblasts, in which the parts characteristic of the trochophore are reduced to a mere rudiment, and the entire trunk develops on the surface of the egg. Between these two extremes come the other forms mentioned, and it may be said in general of them, that there is a gradual acceleration in the time of development of the trunk very nearly in proportion to the increase in relative size of the somatoblasts. Treadwell has elsewhere called attention to part of this series, and has come to the conclusion that "the extra amount of material stored in cell *D* of *Nereis*, *Amphitrite*, etc., is in some way related to the need for an extra amount of somatic and mesoblastic material in the young larva."

IV.

But in order to show that the adaptation need not run in the one direction of the trunk, let me cite one more case, which I am able to add through the kindness of Dr. E. B. Wilson, from some unpublished observations of his on the cell-lineage of a Nemertean. Here the four upper cells of the eight-celled stage are larger than the four surrounding the vegetative pole, the only case of this sort known, I believe. We should expect on *a priori* grounds that the resulting larva would possess a large helmet in front of the ciliated band, which marks the posterior boundary of the products of the first quartet of ectomeres, with a hardly developed trunk region and a rudimentary archenteron. Such is, in fact, the case. The enormous development in the pilidium of the exumbrella is predelineated in the unique third cleavage.

I do not believe that adaptation is one whit less far-reaching in the cleavage than in the larva. In fact, if the principle which I am defending be correct, the two must be coextensive; that is to say, adaptation in cleavage is no more caused by

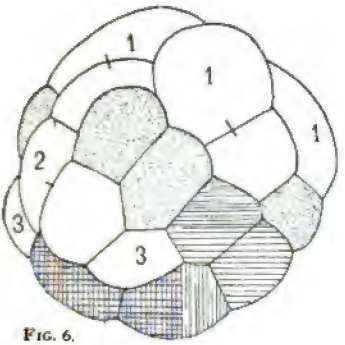

FIG. 6.

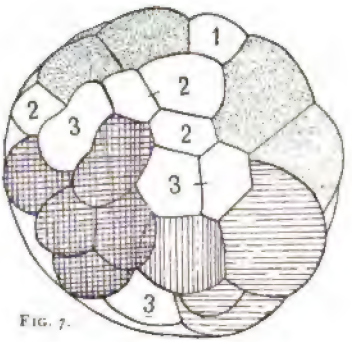

FIG. 7.

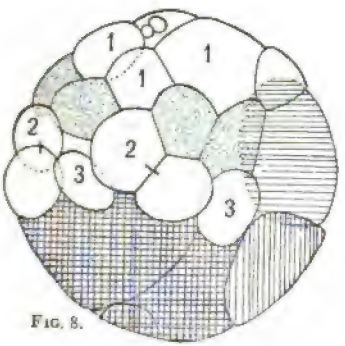

FIG. 8.

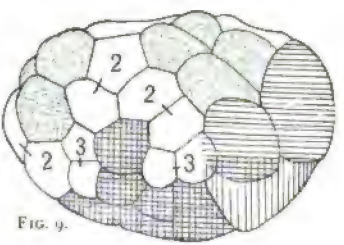

FIG. 9.

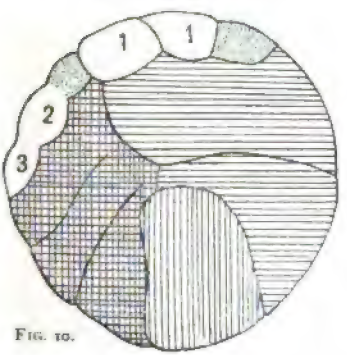

FIG. 10.

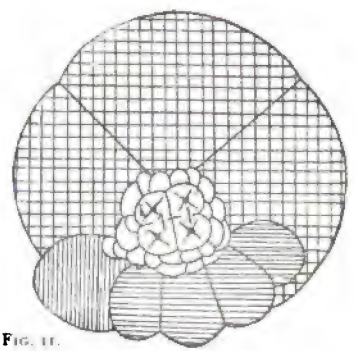

FIG. 11.

FIGS. 6–11. — Cleavage of a number of annelids, showing progressive increase in the size of the trunk-forming cells, *viz.*, the first and second somatoblasts, $d^2 = X$ and $d^4 = M$. The first somatoblast, which forms the ectoderm of the trunk, is indicated throughout by horizontal lines, and the second somatoblast, which forms the mesoderm of the trunk, by vertical lines. The endoderm-cells are ruled with lines crossing at right angles. The stippled cells are the primary trochoblasts, destined to form the prototroch. 1. First generation of ectomeres; 2. Second generation of ectomeres: 3. Third generation of ectomeres.

FIG. 6. — Thirty-two-celled stage of *Podarke* from the left side. Taken, with the kind permission of Mr. Treadwell, from one of his unpublished drawings.

FIG. 7. — *Amphitrite*, after Mead. From the left side and below.

FIG. 8. — *Nereis limbata*, after E. B. Wilson, from the left side.

FIG. 9. — *Clymenella torquata*, after Mead, from the left side.

FIG. 10. — *Aricia*, optical longitudinal section, after E. B. Wilson.

FIG. 11. — *Clepsine*, after C. O. Whitman. From the animal pole. The great size of the endoderm-cells is due to yolk. The somatoblasts far exceed in bulk all the other cells.

adaptation in the larva than *vice versa*, but the adaptation in all stages of the development is due to some common cause. It is, of course, impossible to be blind to the fact that this "explains" determinate cleavage only in a very limited sense. But if the principle serves to restrict the too universal application in the cleavage of the egg of the so-called "mechanical laws of cell-division," much will have been accomplished.

V. Protoplasmic Basis of Adaptation in Cleavage.

It is possible to explain variations in size, position, and rate of cleavage of the cells, by the hypothesis of differentiation of the cytoplasm into materials of different qualities and positions (His, Whitman *et al.*).[1] A much more difficult question is immediately suggested, namely, How do these different substances arise within the cell, and how are they distributed to definite regions? Are all of the differences that exist in the thirty-two-celled stage, for instance, also to be found in different substances of the unsegmented ovum, or are they formed, wholly or in part, during the cleavage, and, if so, how?

The following observations, selected from my work on the fertilization and first two cleavages of the egg of *Unio*, seem to throw some light on this difficult problem. The spermatozoön enters always at the centre of the vegetative pole, and describes a penetration-path that carries it to a point between the centre and the periphery of the egg near the lower boundary of the upper (animal) half. The penetration-path is marked by a portion of the cytoplasm cleared of yolk-granules by the activity of the sperm-amphiaster (Fig. 12). During its penetration the sperm-head has formed some caryolymph, and hence is somewhat vesicular; but, arrived at its region of rest, it contracts into a clump of small chromatic elements. All this has taken place during the formation of the first maturation-spindle, and is completed before the cytoplasm of the animal pole begins to protrude to form the first polar globule. The sperm-amphiaster and visible penetration-path have disappeared by the time of

[1] A theory of polarization of the ultimate morphological units would afford only a partial explanation of the differences in rate and direction of cleavage and in the size of the cleavage products.

the metaphase of the first maturation-spindle, and the sperm-nucleus remains absolutely quiescent during the remainder of

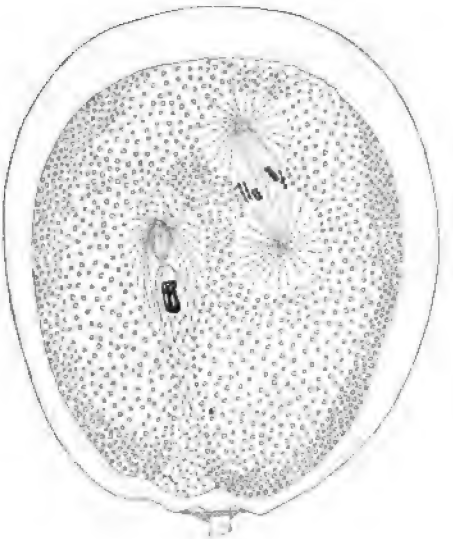

FIG. 12. — Early fertilization of the egg of *Unio complanata* in actual section. The space between the membrane and the egg proper has been reduced to economize space; the micropyle, penetration-path of the spermatozoön, sperm-amphiaster, and first maturation-spindle are shown. A large proportion of the yolk-granules is temporarily forced to the periphery.

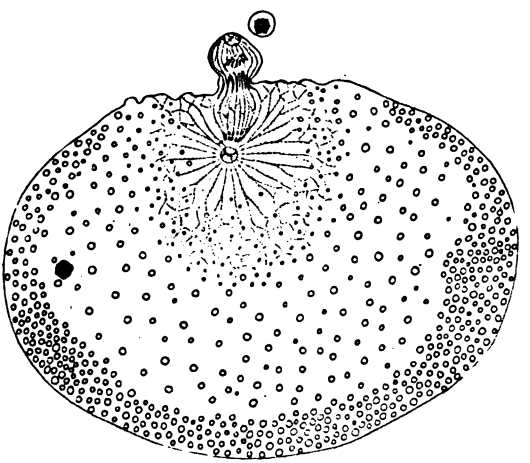

FIG. 13. — Formation of the second polar globule in *Unio*. Sperm-nucleus to the left.

the maturation processes, until the egg-nucleus begins to grow. Let us now study the egg during the final constriction off of

the second polar globule. At this time (Fig. 13) the sixteen chromosomes that are to remain in the egg are closely packed together near the animal pole, and are almost in contact with the inner sphere of the aster. The inner sphere is derived from the centrosome of the first maturation-spindle (see No. 8) and has the following structure: it is a spherical body with a perfectly definite wall, containing a non-staining substance in which is excentrically placed a single centrosome united to the wall by a few strands. The cytoplasm in the immediate vicinity is arranged in the form of an aster, the fibres of which are centrally attached to the inner sphere; the first row of microsomes on the radiations of the aster bound a fairly definite outer sphere. The sperm-nucleus occupies the same position as before, and has undergone no change of structure; in Fig. 13 it is represented lying to the left. During the final stages of formation of the second polar globule, the inner sphere begins to enlarge, and the interior is occupied by a reticulum, in the nodes of which are a number of centrosome-like bodies. After the second polar globule is fully formed, the sphere enlarges with great rapidity (Fig. 14), and, as its boundary spreads out, it becomes less and less definite, until its substance merges with the general cytoplasm. During its enlargement, the interior is occupied by a vesicular substance, at the nodes of which are deeply staining granules, in no wise distinguishable from microsomes. The substance of the inner sphere is now, in fact, part of the general cytoplasm. Yolk-granules are entirely absent in it (Fig. 15), and this enables one to follow its subsequent fate for a considerable period of time.

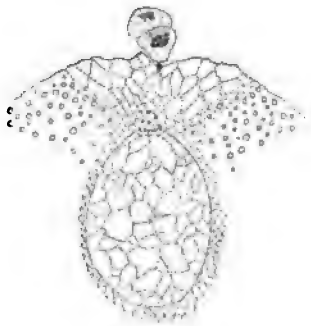

FIG. 14. — *Unio*. Small portion of the upper pole. Egg-nucleus reconstituting; sphere-substance forming.

We shall call this substance, provisionally, *sphere-substance*, following Conklin (4); it is important to remember that it is derived entirely from the inner sphere of the second maturation-spindle. Either this is the case, or else the apparent enlargement of the inner sphere at the close of the maturation is due

to a wave of condensation in the cytoplasm, travelling outwards, sweeping away the yolk-granules, but leaving the cytoplasm behind. The latter theory would explain the appearance of vesicular cytoplasm within the boundary during the phenomenon, and so avoid the difficulty of explaining the extremely rapid growth of substance within the sphere; it could be compared to the wave that spreads out from a pebble dropped in the water. But it is difficult to understand the nature of such a disturbance. On the whole the *expansion* theory seems to me much more probable than the *wave* theory, and the latter is certainly untenable in the first slow stages of the process.

Another important thing to notice is that just before the expansion begins the sphere is three-quarters surrounded by the chromosomes, and I would like to hazard the conjecture that, at this time, there may be a diffusion of some chromatin substance within the sphere, the interior of which tends to stain more darkly than before. If this be actually the case, it has an extremely interesting bearing on subsequent events.

Let us now trace the further actions of the germ-nuclei and sphere-substance during the first cleavage. In general, what I propose to show is that the sphere-substance moves and elongates so as to mark out a definite horizontal plane in the egg, and that the first cleavage-spindle places itself in conformity with this predetermined arrangement.

Each of the germ-nuclei, at the close of the maturation, is a small, dense clump of chromosomes, and both begin to swell up into the vesicular form of the ordinary resting nucleus at the same time, and keep step throughout the process. This indicates a new general condition of the egg-cytoplasm; for, while we might explain the enlargement of the egg-nucleus alone as part of the usual sequence of mitosis, due to purely localized conditions of the egg-substance, we can only explain the effect simultaneously produced on both nuclei by the assumption that the entire egg-cytoplasm is entering on a new phase of its development.

The first movements of the germ-nuclei begin after each has enlarged considerably. In its early movements the egg-nucleus is preceded by the sphere-substance, which moves towards the

centre of the egg, and *always towards the side opposite to that in which the sperm-nucleus lies* (Fig. 15). Thus the egg-nucleus first moves away from the sperm-nucleus, which immediately takes up its march in an amœboid manner towards the centre of the egg, and in the general direction of the egg-nucleus. I may finish at once with the movements of the germ-nuclei by saying that they ultimately come together in the centre of the egg. Whether or not the first movement of the egg-nucleus away from the sperm-nucleus is passively brought about by the sphere-substance, the behavior of the germ-nuclei shows that their meeting is not entirely due to mutual attraction, but partly, at least, to a common tendency to seek a dynamic centre of the egg.

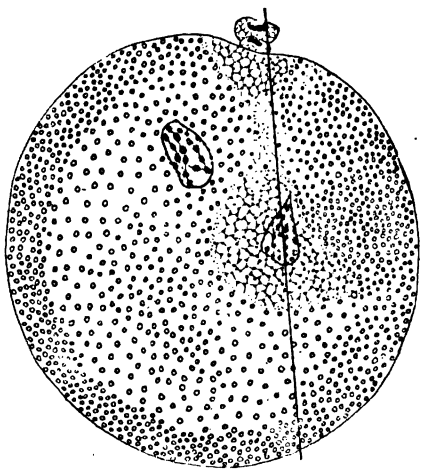

FIG. 15. — *Unio*. Early movements of the germ-nuclei and of the sphere-substance.

To return to the sphere-substance: its invariable migration towards the side of the egg opposite to that in which the sperm-nucleus lies indicates one of two things, — either that the sperm-nucleus has driven it away, or else that it is moving along lines of orientation of the egg-substance. To suppose that the minute sperm-nucleus could exercise such an effect seems impossible, and the corollary of the second alternative is that the sperm-nucleus has occupied throughout its entire resting period a definite position within the egg, which can only be explained on the assumption of a definite orientation of the egg at the time of fertilization, not only polar, but also corresponding to one of the other chief axes of the embryo. The first cleavage-plane passes very nearly through the point at which the sperm-nucleus has been resting during the maturation.

During its migration towards one side of the egg, the sphere-substance has undergone a remarkable change of form; it has

elongated greatly in a horizontal direction, at right angles to the line uniting the germ-nuclei (Fig. 16), *and has thus marked out the direction of the first cleavage-spindle. Now this elongation has begun, and the plane of division is indicated before the germ-nuclei have met.*

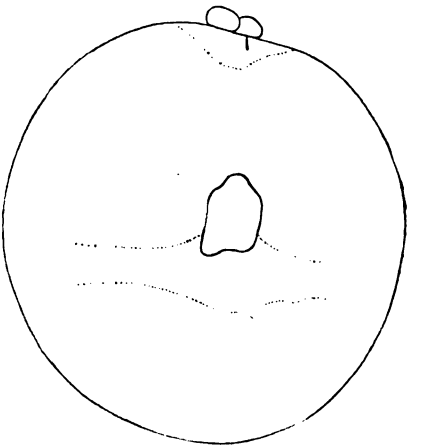

FIG. 16. — *Unio*. Outline of section in the direction of the line ruled across Fig. 15; to show the elongation of the sphere-substance.

It might very readily be assumed that the plane of the first cleavage is determined by the copulation-path of the germ-nuclei, as is stated to be the case in the ova of some other animals (frog, Roux; *Toxopneustes*, E. B. Wilson). But whoever should take this position for *Unio* would have to explain how it happens that the sphere-substance elongates in the plane of the first cleavage-spindle before the germ-nuclei come together. It would be necessary, I believe, to assume that the distant sperm-nucleus exercises an influence on the direction of migration and of elongation of the sphere-substance in the first cleavage, but that, in the next division, the sphere-substance acts independently; and this assumption is absurd on the face of it.

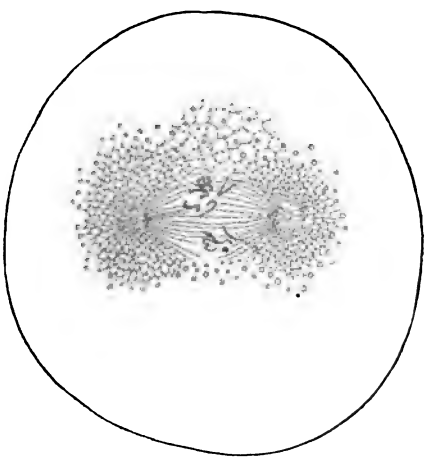

FIG. 17. — *Unio*. First cleavage-spindle forming. Basichromatin granules near the ends of the spindle; oxychromatin granules on the spindle-fibres.

The first cleavage-spindle forms in the centre of the egg (Fig. 17), in the plane already indicated by the elongation of the sphere-substance. At first there is a single very minute

centrosome at each pole, almost in contact with the nuclear membrane, then a group of centrosome granules imbedded in a ground substance; then comes a clearing of the centre, accompanied by peripheral arrangement and subsequent fusion of all the centrosome granules, excepting one, which remains in the centre. Thus is established a hollow sphere ("centrosome") with an included centrosome ("centriole") (Fig. 18). During the early stages of the metamorphosis of the centrosomes, chromatin granules from the nucleus are found in their immediate neighborhood, and, as the spindle forms, they become closely pressed to the centrosomes (Fig. 17). The entire spindle then moves directly along the prolongation of its axis, thus parallel to the direction of elongation of the sphere-substance, to one side of the egg (Fig. 18), until the centrosome of one end comes almost in contact with the peripheral layer of protoplasm. Then comes the metaphase (Fig. 18), and, in a late stage of the anaphase, the sphere begins to enlarge, and a reticulum (or vesicular substance) with nodal microsomes (Fig. 19) develops in it in place of the single centrosome.

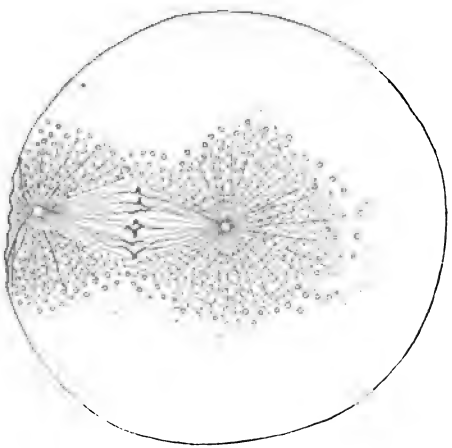

FIG. 18. — Migration of the spindle to one side of the egg; metaphase; for the sake of clearness only a few of the chromosomes were drawn in.

The egg now begins to elongate at right angles to the plane of division, and the entire spindle, including the enlarged spheres, shifts towards the centre of the egg a short distance, and finally comes to rest (Fig. 19). The spindle acts as though oscillating through a point of equilibrium, at first shifting too far in one direction (Fig. 18), then swinging back (Fig. 19), and possibly undergoing lesser shiftings before coming to rest for the first cleavage.

To what extent this centre of equilibrium is determined by the primary orientation of the cytoplasm, and how much by the secondary distribution of the sphere-substance, it is impossible to say; but the orientation of the cytoplasm is, in either event, the primary factor, inasmuch as it determines the distribution of the sphere-substance.

The first cleavage-furrow now forms rapidly, and, as it forms, the spheres undergo an enlargement, migration, and change of shape analogous to that preceding the first cleavage. We shall follow it only in the larger cell. The sphere-substance

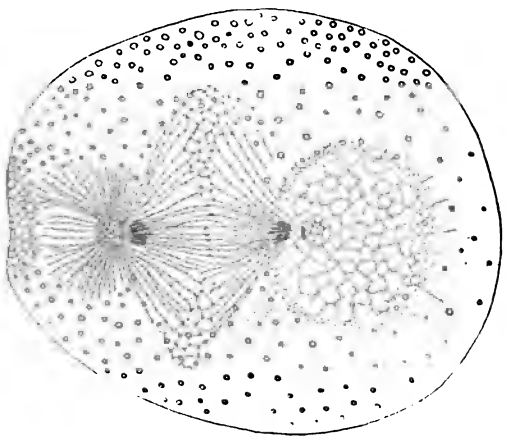

FIG. 19. — *Unio*. Secondary shifting of the first cleavage-spindle. Beginning of growth of the sphere-substance.

in this cell elongates during the early stages of reconstitution of the nucleus in a horizontal direction parallel to the axis later taken by the second cleavage-spindle in this cell (Fig. 20). At the same time the entire cell elongates in the same direction and becomes slightly constricted in a plane and position corresponding precisely with the next cleavage-plane; this, before the nucleus is reconstituted, or has moved away from the neighborhood of the first cleavage-wall. It is as though the cytoplasm were making an attempt at division, which is rendered abortive by the stage of development of the nucleus. Later both cells round off and then become applied together, and the second cleavage-spindle forms and moves into the position of the division already indicated.

Conklin (4) was the first to call attention to the possible importance of the sphere-substance in the cleavage of the egg. He summarizes his results on the egg of *Crepidula* thus : "After the first two cleavages the sphere-substance is differently distributed to the different cells, the entire sphere-substance of one generation always going into those cells of the next generation which lie nearest the animal pole. This differential distribution of the spheres has been followed through every cleavage up to the twenty-four-cell stage. As the form of the cleavage is perfectly constant, it follows that the sphere-substance of any generation goes into certain definite cells which have a perfectly constant origin and destiny. This differential distribution of the spheres is not caused by their specific weight, since their movements are the same in whatever position the cell may be placed. It seems to be the result of a form of polarity which, like that of the egg itself, is not the result of gravity.

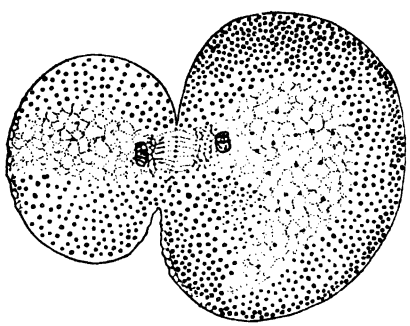

FIG. 20. — *Unio*. First cleavage; the elongation of the sphere-substance in the larger cell and of the cell itself marks the plane of the second cleavage. In a slightly later stage there is a well-marked constriction across the cell in the position of the future second cleavage-spindle in this cell.

"The centrosomes do not, apparently, arise from the sphere-substance of the previous division, but some distance from it, and the sphere-substance never divides, but each sphere ultimately grows ragged at its periphery and gradually fades out into the general cytoplasm.

"The differential distribution of the spheres and their subsequent conversion into cytoplasm suggest that they may be important factors in the differentiation of cleavage cells, and if further investigation should establish the fact that they are, in part, composed of the oxychromatin of the nucleus, it would furnish a basis in fact for certain well-known speculations of de Vries, Weismann, and Roux."

Thus, putting Conklin's results [1] on the movements of the sphere-substance and my own together, it would appear that, whereas in the first two cleavages this substance is divided between the cells in proportion to their size, in the formation of the generations of ectomeres the substance enters special cells. This would coincide very closely with the differential value of the cleavages, the first four cells possessing ectoblastic, entoblastic, and, in part, mesoblastic materials, while the three subsequent divisions of the macromeres separate ectoblastic portions. This tends, it seems to me, to strengthen Conklin's conclusion that the sphere-substance may be an important factor in the differentiation of cells.

Finally, I do not believe that the process of nuclear or cell-division is ever in itself an act of differentiation. That it is not, in certain cases at any rate, is shown beyond the possibility of any doubt by examples of non-determinate cleavage, such as that of the fish-egg, in which the cleavage-planes bear no constant relation to each other or to the embryonic parts, and, still more strikingly, in the case of ciliate *Infusoria*, where the entire process of development takes place without any cell-division. If my observations are correctly interpreted in what has preceded, the essential process in early embryo-formation proceeds on the basis of a definite orientation and organization of the egg-substance, carried forward and elaborated by certain intercellular processes, in which the production of special substances which have been acted on by the chromatin may play an important rôle. Now the distribution of these substances is not dependent on cell-division, though by this they may be isolated in separate cells; but it is conceivable that the cleavage-planes may, so to speak, ignore the lines of orientation of the egg and of distribution of specific parts of it; thus it may be that determinism in the cleavage is no measure of the degree of organization of the egg, as Whitman has so ably argued.

It is quite possible that there is no sharp distinction between

[1] In *Crepidula*, apparently, the substance of the spheres is not divided in the second cleavage, but passes into special cells. See lecture by Dr. Conklin in this volume.

determinate and indeterminate cleavage, and that one grades into the other, the apparent difference being due to insufficient knowledge, as Conklin and Eisig (5) have suggested. But there can be no doubt in the mind of any one as to the existence of a very real difference between determinate and indeterminate types of cleavage, who has compared, for instance, the cleavage of the egg of an annelid, possessing a perfectly definite and unvarying mode of cleavage and cell-lineage of organs, with that of a fish, in which slight alterations of the external conditions cause the very greatest variations in cleavage, so that often the cells of two eggs of the same species cannot be homologized, and no definite cell-lineage of organs exists. The explanation of this difference, it seems to me, is a prospective one. It is dependent, I believe, on the actual number of cells composing the embryo at the time that the first larval or embryonic organs come into service. In other words, I would think of determinate cleavage as an adaptation to a condition in which the functional activity of organs begins with a relatively small number of cells, and in which, therefore, each cell is of special importance.

MARINE BIOLOGICAL LABORATORY,
August, 1898.

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SOME NOTES ON REGENERATION AND REGULATION IN PLANARIANS.

FRANK R. LILLIE.

I. The Source of Material of New Parts and Limits of Size.

Many observers have noted the tendency of planarians kept without food to diminish in size. My attention was specially directed to this phenomenon by some experiments undertaken to test the effect of external conditions on the regeneration of *Planaria maculata*. I had already studied the effect of temperature on the regeneration of this form in conjunction with Mr. Knowlton.[1] I next undertook to test how far the chemical constitution of the medium affected the rate and form of regeneration. As an introduction to the systematic study of this subject, I made some experiments to determine whether any of the substances dissolved in the water of the habitat is necessary for regeneration. For this purpose I redistilled some of the ordinary distilled water of the laboratory, using flasks of Jena glass to get rid of the minute traces of copper found in water from copper stills. Permanganate of potash was dissolved in the water in the Jena flask to destroy traces of organic matter, and the distillate was again distilled in the same manner to insure the greatest possible degree of purity. The redistilled water thus obtained was carefully oxygenated by running a stream of air through it.

The pieces of planarians used for the experiments were washed in this water and then transferred to more of the same; the vessels (cleaned in strong acid) and water were changed frequently to get rid of any traces of bacterial growth. I soon found that under these circumstances regeneration went on as

[1] Lillie and Knowlton. The Effects of Temperature on the Development of Animals, *Zoöl. Bull.*, vol. i.

rapidly and as well as in tap water or the water of the habitat; thus demonstrating that the substances contained in these waters were not necessary for regeneration.

However, the decrease in size already mentioned was so rapid and marked as to appear to deserve special study. So I isolated a number of active planarians in the redistilled water, measured them, and kept them in a thermostat at temperatures that ranged from 20° to 27° C. The dishes and water were changed regularly and frequently, measurements of each individual being made at the same times. The table on the opposite page shows the history of these specimens; less than half of the actual measurements in this series are given.

It will be noted that the rate of decrease was not the same in all; in 2, for instance, it was much more rapid than in the others. This is probably due in part to greater activity of 2. Nor is the rate perfectly uniform in any given specimen, probably owing to variations in the temperature and in activity at different times.

The smallest specimen obtained (No. 5, after 43 days) was certainly less than one-hundredth the bulk of the original animal. Its length was one-fifteenth the original length, its width one-third the initial width, and if we suppose that its dorso-ventral diameter was reduced by only one-half, the bulk would be one-ninetieth of the original bulk. But there can be little doubt that its dorso-ventral diameter was reduced more than one-half.

Increase by fission was entirely stopped, but, on the other hand, the power of regeneration remained. Thus, when No. 5 had been reduced to less than one-half its original length, it was cut in two parts, and both regenerated completely, *although with constant diminution in bulk*. The same experiment succeeded in No. 3, after it had been starved to less than one-fourth its original length.

It is thus demonstrated that from a given individual one of less than one-hundredth the original bulk may be produced by appropriate means. This constitutes a criticism on those experiments that have been made to determine the limits of regeneration in planarians by direct operation. The possible

Days.	1.	2.	3.	4.	5.
1	7 × .7	7 × .5	10 × 1	6 × .5	9 × .75
5	7 × .7	5.5 × .5	7 × 1	6 × .5	8 × .75
12	6.5 × .5	4 × .33	6 × .66	Dead.	5 × .5
17	6 × .5	4 × .33	5 × .4		4 × .3
23	4 × .5	2.5 × .5	4.3 × .5		3.25 × .3 Cut in two.
25		Cut in two.			1.75 × .3 (head) 1.25 × .3 (tail)
27	3.7 × .4	1.25 × .3 (head) Partial regeneration. Died.	4 × .4		1.75 × .3 (head) 1.25 × .3 (tail) Partial regeneration.
33	3 × .4		3 × .4		1.4 × .3 (head) .9 × .3 (tail) Complete regeneration. Anterior piece died two days later.
40	2.3 × .4		2.7 × .4		.6 × .25
43	1.9 × .4 Cut in two. Parts died in two days.		2.25 × .4 Cut in two. Tail end, 1.1 × .4; regenerated in four days (partly). Head end, .9 × .4; regenerated very little new tissue.		.6 × .25 Lost by accident.

Table giving history of five planarians in distilled water. The vertical columns should be read separately. There is a slight discrepancy in the measurements of 2 and 3 for 17 and 23 days, no doubt due to an error in observation. The column to the left gives the number of days from the beginning of the experiment. The measurements, made after 17 days with the ocular micrometer, have all been reduced to millimeters.

limits of regeneration have been found by such experiments to be not much less than one-sixteenth of the bulk of the original animal. That a much lower limit has not been found is due partly to the extensive exposure of internal tissues along the cut surfaces, and partly (perhaps) to limitation of variety of tissue, but certainly not, it would appear, to deficient size.

Do all organs suffer equally in the reduction? It might be expected that the organs of reproduction would suffer first, as they do not contribute to the life of the individual; but the specimens used in the experiments were not sexually mature, so this point was not settled. The other systems of organs were reduced in apparently similar proportions. Thus the intestinal diverticula in 5 were reduced to five on each side, and the branches of the longitudinal nerves were apparently equally reduced.

Does the reduction affect chiefly the size or the number of the cells? A careful histological study would be necessary to answer this question in detail. Not having made this, all that I can say is that the branched pigment cells lying near the surface, that are readily visible under a low magnification, are reduced to very few, but their size is not noticeably affected.

Do the processes of reduction retrace the steps of normal growth and development? I think that this question must be answered in the affirmative. Certain it is that specimens reduced by starvation to a smaller size than just hatched specimens of the same species resemble these in their general proportions, the relatively greater breadth in proportion to length as compared with mature specimens, the smallness of the cephalic lobes, and in the small number of the intestinal diverticula and branches of the longitudinal nerves. It would be interesting to determine whether or not these artificial embryos, as they might be termed, could under favorable circumstances repeat the steps to the mature and fully grown condition from which they were reduced. I see no reason to doubt that this is possible.

In regeneration, under the circumstances of these experiments, two processes are taking place side by side; not only is new tissue being formed at the cut end, but the old tissues are

undergoing a translocation and partial redifferentiation to accommodate themselves to the new proportion that must be assumed. The new tissue at the cut end must be formed entirely from the old tissue, and the final result involves, therefore, an extensive working over of the old material. The original tissues, with constant losses, owing to destructive metabolism, are moulded into the form of a new individual. In trying to form a mental image of the forces at work in this complex rearrangement, one can think only of an "internal mould" (an expression of Buffon to which Professor Whitman has called my attention). No less remarkable is the maintenance of form in individuals that gradually waste away to one-hundredth part or less of their original bulk. Such individuals in all stages of reduction appear normal in all respects, physiologically as well as morphologically. They are active and exhibit perfectly normal reactions, showing no indications of sickness.

VASSAR COLLEGE, Jan. 4, 1900.

THE ORGANIZATION OF THE EGG OF UNIO, BASED ON A STUDY OF ITS MATURATION, FERTILIZATION, AND CLEAVAGE

BY

FRANK R. LILLIE

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INTRODUCTION.

THIS piece of work was originally undertaken in 1896 as a study of the behavior of the centrosomes in the fertilization of the egg of *Unio*, but I was soon led into a *general* study of the centrosomes in this egg. The results which I obtained seemed to be irreconcilable with the views then current of the functions of the sperm-centrosomes in fertilization, and with

the general theory of the permanence of the centrosome as the special organ of cell division, or the center of organic radii. Brief abstracts of the results were published in the form of two short papers, read before the American Society of Morphologists in the winter meetings of 1897 and 1898, summaries of which appeared in *Science* in March, 1897 and 1898. The second paper was also published in full in the *Zoölogical Bulletin*, Vol. I. Part of the subject was described for the first time in my paper on "Adaptation in Cleavage," in the *Biological Lectures* for 1898 (Lillie, '97–'99).

Since this work was begun the entire centrosome question has entered on a new phase of development, and the subject now appears to me to be in such a condition that the great need is for careful observations rather than for any attempt to found new theories or defend old ones; any such attempt in the present state of our knowledge would be premature. It is in this spirit that I publish the following detailed account of my observations. The work, moreover, has had for me a greater embryological than purely cytological interest; hence I lay the greatest weight on the evidence as to the organization of the egg. The conclusions concerning this subject link themselves directly to my earlier observations ('95) on the cell-lineage of *Unio*, and are a further elaboration of the principles laid down in that paper.

The egg of *Unio* is most definitely oriented. The orientation is not due to the arrangement of the deutoplasmic materials, for the yolk-granules have no definite abiding-place in this egg, but are driven here and there by cytoplasmic movements. Yet of all the many hundreds and even thousands of eggs examined, not one was found in which the polar globules were not formed opposite to the micropyle, which thus occupies the center of the vegetative pole. It is here, of course, that the spermatozoön enters.

The eggs are fertilized in the suprabranchial chamber by spermatozoa that enter with the inhalent current of water. Apparently, contact with the water may act as stimulus for the first breaking of the germinal vesicle; because, so far as my observations go, the spindle is not formed in the ovary, and I

have found unfertilized eggs with the first maturation spindle fully formed. Moreover, I have never found an egg, into which the spermatozoön had entered, in which, at the same time, the first maturation spindle was not forming.

It seems probable, from some observations I have made on the breeding habits of *Unio*, that the presence of spermatozoa in the water may act as a stimulus to the female, causing the extrusion of eggs.

The general course of events after the entrance of the spermatozoön is this : a sperm-amphiaster is formed very soon after the penetration of the sperm-head, which moves up towards a definite zone above the equator of the egg and there comes to rest. The sperm-amphiaster gradually disappears, and the sperm-nucleus, which has enlarged considerably during its penetration, contracts in volume nearly to its original bulk. By the time that this is accomplished the first maturation spindle is entering on its metaphase. The sperm-nucleus undergoes no further changes until after the completion of the second maturation division, at which time it grows in size synchronously with the egg-nucleus and begins to describe its copulation path. The copulation path of the germ-nuclei is by no means simple, but they ultimately come into contact at or near the center of the egg. The first cleavage spindle is then formed, and moves axially to one end of the egg ; thus the first cleavage is very unequal.

I. The Behavior of the Spermatozoön in the Egg up to the Time of Extrusion of the Second Polar Globule.

1. *The Sperm-Nucleus.*

The spermatozoön enters through the micropyle shortly after the breaking down of the germinal vesicle. In mounts of entire eggs at this stage one can see a number of spermatozoa clustered around the region of the micropyle, and may even see one within it. But it very rarely happens that more than one enters and fuses with the egg, for I have not seen

more than two or three cases of polyspermy in the thousands of eggs examined. The head of the spermatozoön is shaped like a blunt cone, and so should furnish evidence of rotation, if this really occurs. But as it rounds off almost immediately on entering, and as the sperm-aster does not form at once, the evidence is inconclusive.

The usual path of the spermatozoön is shown in Pl. XXIV, Figs. 1 and 2. It is always towards one side of the egg and in the direction of the animal pole. The path is frequently quite direct, as in Pl. XXIV, Figs. 1 and 2, but is often curved more or less. When the sperm-nucleus has passed above the equator a little way, its movement stops and it remains quiescent until after the formation of the second polar body.

During its penetration the sperm-nucleus swells up somewhat by the accumulation of caryolymph within it, forming a nucleus with a thick chromatic wall and a reticulum of a few strands only. At this time it possesses almost invariably a sharp apex directed forwards and a broad base, thus corresponding very nearly in form to the head of the spermatozoön before its entrance. But as it has power, after entering, to change its form very considerably, it does not of necessity follow that the apices correspond in the two cases.

Arrived at a point of equilibrium in the egg-substance, its place of rest, the sperm-nucleus rounds off and contracts in volume. As it does so, the chromatic reticulum becomes very regular (Pl. XXIV, Figs. 7, 8, 9 *a* and 9 *b*). By careful study it is possible to determine that there are about sixteen regular hexagonal interchromatic areas on the surface; and as this agrees with the number of the chromosomes that ultimately develop from it, it seems probable that each of these areas corresponds to a chromatin-vesicle or chromosome. The contraction proceeds during the anaphase of the first maturation division, until the cavity of each vesicle becomes indistinguishable, and the sperm-nucleus is then a small mulberry-like body, staining intensely black. Sobotta ('97, p. 46) has described in *Amphioxus* a similar enlargement and subsequent contraction of the sperm-nucleus.

There is nothing specially worthy of note in the further behavior of the sperm-nucleus during its resting period; apparently it undergoes no further change, and certainly makes no other movements until after the formation of the second polar body. Then it begins to enlarge again by the accumulation of caryolymph in its interior, keeping pace with the growth of the egg-nucleus; and both nuclei begin to move inward at the same time. The details of the growth and movements of the germ-nuclei are better considered together, and will be described thus after the section on the maturation of the egg.

2. *The Sperm-Aster and Amphiaster: Origin and Disappearance.*

a. *Observations.* — I have not been able to determine the exact origin of the sperm-centrosome in the egg of *Unio*, and have nothing to contribute to this subject. The aster is comet-like in form during the penetration, and possesses a small apical centrosome which soon divides; separation of the halves produces a typical amphiaster. The amphiaster frequently precedes the sperm-nucleus, with its axis in the line of movement and its long rays trailing behind (Pl. XXIV, Figs. 1 and 2) and losing themselves in the cytoplasmic reticulum. But the centrosomes may separate in a direction at any angle to the penetration path up to a right angle (Pl. XXIV, Fig. 4). It seems probable that the latter is the case, when the division of the centrosome is delayed until the penetration path is nearly completed. Frequently the rays of the amphiaster engage with those of the inner aster of the maturation spindle, *and may become continuous with them.* In one case I found an apparently perfect spindle, one pole of which was formed by the inner centrosome of the maturation spindle, and the other by one of the sperm-centrosomes. This would seem to indicate development of the fibers from a common reticulum or foam-structure.

The comet-like form of the aster in the egg of *Unio* seems to be quite unique. That it is not due to any peculiarity in the structure of the protoplasm of this egg is shown by

the fact that the same aster becomes radial after the penetration path is complete. Therefore, it is due in some way to the conditions of the penetration; these probably affect the form of the aster in two ways: 1. It is noticeable that rays form more readily in protoplasm free from yolk-granules; there is a yolk-free path behind the amphiaster, and the rays would naturally be longer within this area. 2. The mere forward movement of the centrosomes would tend to draw out the rays extending behind and to shorten those in front.

Shortly before the metaphase of the first maturation spindle the sperm-asters undergo retrogressive metamorphosis, and, as they disappear, the yolk-granules flow in and gradually obliterate all traces of the clear area. Figs. 7 *a*, 7 *b*, 8, and 9 *a*, Pl. XXIV, illustrate progressive phases of this process. Figs. 7 *a* and 7 *b* are two sections of the same amphiaster two sections apart. The centrosome nearer the sperm-nucleus (7 *a*) is united to the tip of the latter by a delicate fiber, and the radiations originally surrounding it have completely disappeared. The more distant centrosome is still surrounded by a well-defined aster, which, however, in the irregularity of the rays and the number and size of the microsomes on them, shows plain signs of degeneration. In Pl. XXIV, Fig. 8, we have a more advanced stage of degeneration; the nearer centrosome can now no longer be distinguished among the microsomes, and the more distant one is barely discernible, the rays having nearly disappeared; the yolk-granules have encroached on the clear area. In Pl. XXIV, Fig. 9 *a*, neither centrosome can be distinguished, and the clear area is nearly obliterated. In the last stages of degeneration of the aster the centrosome is distinguishable from a cytomicrosome only by its position. When the aster is entirely gone, one sees in its place simply ordinary vesicular protoplasm with nodal microsomes. There can be no doubt of the resolution of the radiations into the cytoplasm; and if the centrosomes do not simply become microsomes, they certainly become indistinguishable from them by any visible morphological character. The view that they persist as definite entities of specific structure and function is not inherently impossible, but there is nothing in the observations to indicate this, and

the subsequent history of centrosomes in this egg gives no support to such an hypothesis.

b. *Literature.* — There are so many observations of the undoubted origin of the sperm-aster around the middle-piece of the spermatozoön that there is a strong presumption in favor of assuming such an origin even when it cannot be directly observed (Wilson and Mathews, '95; Fick, '93; Foot, '98; Van Der Stricht, '98 and others). But the time at which this aster appears varies greatly in different cases. Foot says ('98, p. 51): "In studying the literature I have not been able to find any satisfactory evidence of the appearance of a sperm attraction sphere earlier than the anaphase of the first maturation spindle." It certainly seems a general rule that the radiations do not appear until about this time, however early the spermatozoön may enter. The case of *Unio* is a striking exception to this rule, for here the sperm-aster is formed, and divides and vanishes before this time. In *Arenicola* (Child, '98) the sperm-aster also appears early, just after the first maturation spindle has taken a radial position, but division follows much later.

Disappearance of the sperm-asters and centrosomes has been observed in a number of cases, though the process is not usually described in detail. On account of the importance of this phenomenon I will give rather full citations:

Van Name ('99) states that in *Planocera* he has not been able to learn anything of the subsequent fate of the sperm-centrosomes, "nor to demonstrate any connection between them and the cleavage centrosomes." From this I conclude that they disappear before the first cleavage spindle is formed.

In *Arenicola* (Child, '97) the sperm-amphiaster is well developed during the last phases of formation of the second polar body. After this is formed "the two centrosomes have disappeared, the polar regions of the spindle being occupied by a fine network. The rays are evidently disintegrating, and the spindle is barely indicated in the cytoplasm. These structures present this appearance with regularity at this stage. It is certainly not due to imperfect fixation or errors in technique, for on the same slide are eggs in somewhat earlier stages, with

distinct 'male' centrosomes, spindle, and radiations. The following stages afford a further confirmation of the view that this disintegration is a part of the normal history of these structures, for a little later there is no trace of asters or spindles in this egg." Concerning the cleavage centers, Child says: "I am inclined to regard the cleavage centrosomes as new formations and as not related to the 'male' centrosomes."

Wheeler ('97) finds no sperm-aster in *Myzostoma*, and though Kostanecki ('98) has since observed its appearance exceptionally in this egg, he has not been able to trace it into the first cleavage spindle. Wheeler finds that the cleavage centers are furnished by the egg.

In *Pleurophyllidia* (MacFarland, '97) the sperm-asters disappear entirely during the formation of the second polar body. The centrosomes can no longer be distinguished.

In *Allolobophora foetida* "the sperm attraction sphere is present until the head of the spermatozoön begins to develop into the male pronucleus, when it also totally disappears. Both spheres, *i.e.*, sperm and egg spheres, are absent during a relatively long period (*i.e.*, while the young pronuclei are developing); and when the pronuclei have attained their maximum size and are in contact, two attraction spheres appear again in the cytoplasm, and the cleavage spindle is formed." (Foot, '97, p. 811).

In *Prostheceraeus* (Klinckowström, '97) both egg and sperm asters disappear completely before the union of the germ-nuclei.

In *Cerebratulus* (Coe, '99), "after the germ-nuclei are nearly in contact, the sperm-asters have served the purpose for which they were intended, and completely disappear in a manner quite similar to that of the aster remaining in the egg after the formation of the second polar body. Even the centrosomes are lost from sight in most cases." Exactly the same thing is true of *Physa* (Kostanecki and Wierzejsky, '96).

In Echinoderms the sperm-asters become much less distinct at the time of meeting of the germ-nuclei. Coe states ('99, p. 455): "Observations which I have made on the eggs of *Echinus*, of *Sphaerechinus*, and of *Strongylocentrotus*, lead me to

believe that this diminution of the asters just before the union of the germ-nuclei is much greater than has been described."

II. The Maturation of the Egg.

I have not observed the breaking down of the germinal vesicle; but from the study of ovarian eggs I have found that before extrusion it lies excentrically near the animal pole. In the earliest stage of the first maturation spindle observed (not figured) the two centrosomes lie near the periphery in the neighborhood of the animal pole, and at the same distance from it; the chromosomes lie between them, but much nearer the center of the egg, so that the spindle is bent in the middle. The asters are barely indicated in this stage. The axis of the spindle straightens by the inward migration of the centrosomes to the level of the chromosomes, so that the whole spindle lies very little above the center of the egg. Then follow the rotation, elongation, and peripheral migration of the entire spindle (Pl. XXIV, Figs. 1, 2, 5, 6, and 9).

1. *The Chromosomes in Maturation.*

I have paid relatively little attention to this subject, as I was chiefly concerned with the study of cytoplasmic phenomena. However, some of the observations are of interest. Sixteen chromosomes are formed in the germinal vesicle. In the earliest stage seen they are apparently typical tetrads, which, however, elongate before they become arranged in the equatorial plate. Each chromosome then takes on the form of a longitudinally split rod with two constrictions. The first division is at right angles to the axis of the rod, and passes midway between the two constrictions. The chromosomes take on the very characteristic forms represented in Pl. XXIV, Figs. 1, 2, 5, and 9; material accumulates at the two ends of each chromosome, and, as in a heterotypic division, also in the middle. The chromosome finally parts in the center of this median accumulation. As the chromosomes diverge towards the poles, they undergo typical changes in form, until, when

they reach the poles, each has reassumed the 6-partite form, *i.e.*, a double rod with two transverse constrictions (Pl. XXIV, Figs. 11, 12). The second division of the chromosomes for the formation of the second polar globule is longitudinal, as shown in Pl. XXV, Figs. 19–21.

The forms of the chromosomes in the maturation spindles of the egg of *Unio* resemble closely those in corresponding stages of the eggs of *Prostheceraeus* (Klinckowström, '97), of *Thalassema* (Griffin, '99), and of *Zirphaea*, another lamellibranch (Griffin, '99). The first division is certainly at right angles to the long axis of the chromosomes, as these lie in the equatorial plate, and the second division is with equal certainty longitudinal. But as the history of the tetrads in *Unio* is unknown, it is quite possible that the first division is morphologically a longitudinal division and the second transverse, that is, a reducing division in the usual sense. Klinckowström comes to the conclusion that this is probably the case in the egg of *Prostheceraeus*, though the evidence he offers is not conclusive. Griffin ('99) has made a very detailed study of this form of chromosome, and has shown in an ingenious manner that the first division may be interpreted as following the longitudinal division of the original spireme, and the second as transverse to this, that is, a reducing division in the ordinary sense. If his interpretation should turn out to be just, it would have a wide application, seeing that this form of chromosome is found in flatworms and Gephyrea as well as in mollusks.

2. *Achromatic Structures.*

As already stated, the earliest cytoplasmic phases of maturation have not been observed; thus the question of the origin of the egg-centrosomes will not be considered. We may begin, therefore, with a description of the aster of the first maturation spindle at the time of the metaphase. At this time the aster at either end of the spindle has the following structure (Pl. XXIV, Figs. 9 and 10); in the exact center is a minute centrosome, which has begun to elongate, subsequent to division. This is imbedded in a substance, homogeneous

in appearance, staining uniformly in Bordeaux red, which is traversed by fine radiations centering in the centrosome. A short distance from the centrosome, and at the periphery of the homogeneous substance surrounding it, each ray possesses a distinct microsome; these microsomes together bound a definite body, which I have called *the inner sphere*, and the next row of microsomes on the rays bounds a fairly definite *outer sphere*. Beyond this the radiations pass out into the general cytoplasm for a considerable distance. From the centrosome to the outer sphere each ray is a thread or fiber, but beyond the spheres the rays can plainly be seen to be united by more or less regular anastomoses; and farther out the branching of the rays and the increase in number of the anastomoses cause the rays, as such, to disappear in the general cytoplasmic network. The whole appearance of the aster is such as to give the impression that it has arisen by a central strain in the cytoplasmic network; and the history of the origin of the aster in this and other places in this egg is such as to make this conclusion a practical certainty. I am inclined to believe that the cytoplasmic groundwork in this egg is primarily an alveolar structure, which may become reticular or filar (as in the center of the aster) by the breaking down of alveolar walls. In this opinion I agree with Wilson's conclusions in his recent work (1900) on the structure of protoplasm.

The existence of double rays is a characteristic appearance in this stage of karyokinesis (Pl. XXIV, Fig. 10, and others). However, I do not interpret these as due to "splitting" of rays (Kostanecki, '97), but to approximation of independent rays. One would naturally expect this to occur if the aster arises in the manner indicated.

During the anaphase the centrosome divides into two at each end of the spindle (Pl. XXIV, Fig. 11); and, as the protoplasm begins to protrude for the formation of the first polar body, each one of these four centrosomes becomes double or quadruple (Pl. XXIV, Fig. 12). Another process, which has been going on during the anaphase, is the peripheral accumulation of the ground-substance of the inner sphere and the disappearance in this substance of the bounding row of micro-

somes so that the inner sphere becomes a vesicle with deeply staining wall and clear contents (Pl. XXIV, Figs. 9, 11, and 12). The rays which were originally attached to the centrosomes are now attached to the bounding membrane of the inner sphere. Within the sphere are fibers attaching the centrosomes to the wall, but independent of the radiations of the aster.

When the chromosomes are drawn up close to the inner spheres (Pl. XXIV, Figs. 11 and 12), the functions of the central spindle and asters are for the time at least completed, and both the latter become thickly studded with large microsomes.

Then follows the outpushing of the first polar body (Pl. XXIV, Figs. 12, 13, and 14); as it is being formed, the egg-sphere and chromosomes move up near to the periphery of the egg, at the point of formation of the polar globule. New rays are seen at this time extending from the central sphere to the periphery of the egg; it is probably through the activity of these that the sphere and chromosomes approach the surface (Pl. XXIV, Fig. 13), for the rays disappear as soon as the chromosomes have reached their most peripheral position (Pl. XXIV, Fig. 14). This peripheral transfer of the sphere and chromosomes results in the drawing out of the remains of the original aster into fibers, as shown in Pl. XXIV, Figs. 13 and 14.

During the last phases of the formation of the first polar body there is a very considerable diminution in size of the sphere. This has the effect of bringing the centrosomes much nearer together (Figs. 13 and 14). Sometimes, apparently, they fuse; or the process may go farther, and both centrosomes and sphere may disappear.

The mode of formation of the spindle of the second polar body varies, according to whether the centrosomes and sphere left over from the first division disappear or not. Let us consider the latter case first.

In this case there is within the egg at the close of the first maturation division the sixteen bivalent chromosomes, and a sphere containing two compound centrosomes. This aggregation lies near the periphery of the egg. The aster that origi-

nally surrounded the sphere is still in process of degeneration, and the yolk-granules are beginning to intrude on it (Pl. XXIV, Fig. 14). The fibers that reached from the sphere to the periphery of the egg have completely disappeared. This is a period of inactivity.

Figs. 15–20, Pl. XXV, illustrate the development of the second maturation spindle; they are drawn from eggs in which the spindle formed in its definitive radial position. But in other eggs of the same set the spindle was found forming at various angles with the axis of the egg, later swinging into a radial position. It will be noticed that there is no inward migration of the chromosomes and sphere prior to the spindle formation, as in the eggs of some other animals, and as in those eggs of *Unio* in which the "division-apparatus" of the first maturation division disappears.

The first step in the formation of the new spindle is the enlargement of the sphere; new radiations appear around it (Pl. XXV, Fig. 15); the centrosomes diverge, frequently in a radial direction, as has been already mentioned, and a very minute central spindle is seen between them; the boundary of the sphere still persists. *As will be shown later, the entire inner sphere is the product of a single centrosome; hence in a certain sense "Centrosoma und Centralspindel bilden ein Ganzes"* (Heidenhain).

By elongation of the central spindle the centrosomes are brought in contact with opposite sides of the wall of the sphere, and, pressing against the latter, cause it to elongate (Pl. XXV, Fig. 16). The central spindle, filling the entire interior of the sphere, stretches between the centrosomes, which have become larger and more subdivided, and stain intensely black in iron-haematoxylin. The fibers of the central spindle have been formed out of the ground-substance of the sphere, either by direct conversion of its substance or by growth of the few original fibers at the expense of the ground-substance. The radiations which have arisen anew (Fig. 16) are related to the sphere as a whole, and not to the centrosomes as independent centers.

As the spindle elongates still more, the wall of the sphere

becomes stretched out to form the periphery of the central spindle (Pl. XXV, Fig. 17), and the radiations entirely disappear, leaving the spindle in the center of a mass of vesicular cytoplasm. The outer end of the spindle pushes up through the group of chromosomes nearly to the periphery of the egg. In Fig. 17 the first faint mantle-fibers can also be seen stretching from each chromosome to the inner centrosome.

Fig. 18, Pl. XXV, illustrates a later important phase in the elongation of the spindle. One of the most interesting features of this stage is the change which the inner centrosome has already undergone, to be described in detail immediately below. The elongation of the spindle, after the outer end has become fixed at the periphery of the egg, has resulted in the passive drawing out of the protoplasmic vesicles in straight rows extending to the surface. The appearance of crossing of rays seen in this and other figures is due, I am convinced, to a double strain on the same mass of protoplasm. The chromosomes have now been drawn, apparently by their attached fibers, around the equator of the spindle. The asters are beginning to develop at both ends by continuous arrangement of adjacent alveolar walls. This is very plainly shown by comparison of Figs. 17 and 18, Pl. XXV.

Figs. 19, 20, and 21, Pl. XXV, illustrate successive later stages of the same processes and need not be described in detail here. They carry us to the metaphase of the second maturation spindle. The structure of the centrosome, spheres, and aster in Fig. 20, Pl. XXV, is precisely the same as in the stage with which we started (Pl. XXIV, Figs. 9 and 10). Indeed, at the metaphase of any spindle we have the same structures. *It is always at the metaphase that the centrosome is smallest; at this stage the inner and outer spheres are also invariably marked by concentric rows of microsomes.*

Now, how are these structures related to the stage of Fig. 15, Pl. XXV, with which we ceased our description of centrosome structure? In this stage the large centrosome has simply increased in size and at the same time become more subdivided. Figs. 24–27, Pl. XXV, illustrate the changes which the centrosome undergoes in assuming the form charac-

teristic of the metaphase. These figures represent tangential sections of the outer aster and centrosome in stages corresponding to those of the figures immediately above them, *i.e.*, to Figs. 17–20. To quote from my earlier paper on "Centrosome and Sphere in the Egg of Unio" ('98): "These figures show two processes taking place: (1) the subdivision of the relatively large centrosome granules and their distribution in the form of a sphere; and (2) the increase of the red-staining substance in which the granules are imbedded. The peripherally distributed granules become the stratum of microsomes bounding the inner sphere. One of the granules remains behind as the 'centrosome' of the newer sphere; which one is, apparently, determined entirely by position. The outer sphere has developed during this process.

"The black granules in the inner sphere of Fig. 27 are plainly much less in bulk than those of Fig. 24. There is no doubt that a large part of the centrosome granules has been changed into the red-staining substance of the sphere, which is identical in all noticeable respects with the substance from which the central spindle was formed. In a later stage the fibers of the latter are dotted with large, deeply staining microsomes." Here is evidence of the lack of persistence of the *microsomes* as definite morphologioal elements.

"From this description it would seem to follow that the centrosome of one cell generation becomes the inner sphere of the next; and this is undoubtedly true at times. But I do not believe that the inner sphere has *necessarily* any such definite morphological value as this would seem to imply. For it may disappear between the first and second maturation divisions, and is then re-formed as the first step in the prophase of the second maturation spindle from the cytoplasm. The same method of formation may also be observed in other places (*e.g.*, formation of the first cleavage spindle).

"Both Van Beneden's and Boveri's conceptions of this structure appear as phases in the history of the mitosis, though Boveri's 'centrosome' is really inner sphere, and his 'Centralkorn' or 'centriole' really the centrosome."

I am now, however, inclined to modify the qualification that

the inner sphere has not necessarily a definite morphological value, in the sense that it is always, probably, a product of growth of the centrosome. But it seems to me probable that the latter is of purely functional significance.

"To the criticism that the centrosome phases shown in Figs. 24–27, Pl. XXV, may be pathological, *i.e.*, due to imperfect extraction of the haematoxylin or other action of the reagents, it may be replied: first, that they are found with different killing fluids; second, that the changes are perfectly uniform in all cases, so that, knowing the stage of development of the spindle, one can be certain that a definite stage of the centrosome will be found; third, that inasmuch as the inner aster develops much more rapidly than the outer, the inner centrosome passes through these phases more rapidly. Thus one often finds a spindle, *e.g.*, Figs. 18 and 19, Pl. XXV, in which the inner centrosome has already passed through the entire metamorphosis, while the outer is still in the stage of Fig. 24 or 25, Pl. XXV." Thus one has a typical centrosome at one end and the supposedly pathological centrosome at the other end of the same spindle!

In regard to the formation of the entire central spindle of the second maturation division from the inner sphere ("centrosome," MacF.) my results are in complete accord with those of MacFarland on *Diaullula* ('97). But concerning the origin of the inner sphere ("centrosome," MacF.) of the second maturation spindle I believe my results to be more trustworthy than those of MacFarland. This author describes the inner sphere as arising around the minute "Centralkorn" by a condensation of the substance of the central spindle at either end. I have sometimes found appearances similar to MacFarland's figures in sublimate-acetic preparations which give a poor fixation of the eggs of *Unio*; but when the fixation is good I have always found the centrosomes standing out with the greatest clearness, and their differentiation into the inner sphere with the contained centrosome could be plainly traced. The lack of detail in MacFarland's figures of the cytoplasm gives the impression that the fixation of his materials was not of the best, which might explain our differences in regard to

this point; for it seems to me unlikely that where the agreements are so many such important differences should also exist.

I have already said that the sphere and centrosomes remaining within the egg after the first maturation division may disappear prior to the formation of the second polar spindle. When this happens, the process of formation of the second maturation spindle is (naturally) somewhat different from that already described. There next follows a stage with a single centrosome, whether one of the old ones or not I cannot say; this lies just within and partly surrounded by the chromosomes, and is first recognizable as the center of a small aster. The regular arrangement of the microsomes on the rays of the latter gives the appearance of a regular sphere, analogous, I believe, rather to the outer than to the inner sphere already described. The centrosome divides in two, and the whole combination of chromosomes and aster moves in towards the center of the egg. A minute central spindle can be seen between the two centrosomes, which develops as the latter move apart. The developing spindle takes up a horizontal position central to the chromosomes and a little above the center of the egg (Pl. XXV, Fig. 30). The sphere has dilated and continues to include the entire spindle, although it forms no part of the latter, as in the former method; and the rays of the aster are related to the sphere and do not penetrate within it.

The centrosomes at either end of the spindle now divide repeatedly, forming a group of centrosome granules at either end; and the chromosomes are gradually drawn around the periphery of the spindle at its center. Asters begin to develop at either end, and the whole structure revolves into a radial position (Pl. XXV, Fig. 31), moving towards the surface as it does so. The centrosomes at both ends then simultaneously go through exactly the same process of development as in the former method.

This mode of formation of the second maturation spindle was found in all of the eggs, which were preserved at this stage, of one female; the modification was undoubtedly due

to some slight difference in the constitution of the cytoplasm in the two cases. That such different courses of events can yet lead to the same typical result indicates a most remarkable power of self-regulation of the entire ovum, and at the same time demonstrates that the mechanical processes of spindle and aster formation are not the primary factors in determining the position of the maturation spindles.

The later history of the sphere and centrosomes of the second maturation spindle is shown in Figs. 22, 23, Pl. XXV, and in Fig. 32, Pl. XXVI. During the anaphase the inner sphere becomes hollowed out in the interior, just as in the corresponding phase of the first maturation spindle; and, as there, so here, the wall of the sphere becomes the central area of attachment of the rays, the centrosome itself being united to the wall of the sphere by a few fibers, which are independent of the aster proper. In the stage of Fig. 23, Pl. XXV, the centrosome is invariably placed excentrically within the sphere. What the meaning of this may be I have no idea. The centrosome does not divide during the anaphase of the second maturation division. But in the stage of Pl. XXVI, Fig. 32, the sphere has enlarged considerably, and its interior is occupied by a reticulum, at the nodes of which are a number of microsome-like bodies.

The accessory aster. — Shortly after the metaphase of the second maturation spindle an accessory aster is formed in the egg. It usually arises quite near the center of the egg and bears no fixed relation either to the maturation spindle or the sperm-nucleus; the center of the aster is occupied by an exceedingly minute centrosome. The latter divides and a small amphiaster is formed (Pl. XXV, Fig. 28) which entirely disappears before the stage of Pl. XXV, Fig. 23.

What is the significance of the accessory aster? Is it simply due to a renewal of activity of the sperm-centrosomes which disappeared a cell generation before, or is it an entirely new formation? I do not believe that it has anything to do with the original sperm-asters for several reasons: in the first place, there are two sperm-centrosomes, and the accessory aster is at first single and later divides; secondly, the usual place of

origin of the accessory aster does not correspond with the usual place of disappearance of the sperm-amphiaster; and, finally, in the egg of *Crepidula* (Conklin, '98a) there are two or three accessory asters formed at about this time, while the sperm-aster is perfectly distinct in connection with the sperm-head. Carnoy ('86) has also observed accessory asters of three orders in the egg of *Ascaris megalocephala* during maturation.

This negative result is the only definite conclusion at which I have been able to arrive. It would seem that in this stage the protoplasm of the egg is readily incited to aster formation (*Unio, Crepidula, Ascaris*), and if one cannot refuse the name of centrosomes to the central granule of such asters, it would certainly seem to follow that there are present in the egg other bodies than the sperm- or egg-centrosomes capable of similar function.

III. Growth, Migration, and Union of the Germ-Nuclei; Behavior of the Sphere Substance; Origin of the Cleavage Centrosomes.

1. *Observations.*

Immediately after the formation of the second polar globule the egg-sphere undergoes a most extraordinary enlargement, which takes place so rapidly that it has been almost impossible to find stages intermediate between Figs. 32 and 33, Pl. XXVI. The interior of the sphere is filled with a very wide-meshed reticulum or alveolar structure, in the walls and at the nodes of which are deeply staining granules. The boundary wall of the sphere can be seen during the greater part of the time of expansion; but ultimately (Pl. XXVI, Fig. 34) it becomes stretched beyond the limits of visibility, and the sphere becomes directly continuous with the general cytoplasm. There is thus between the egg-nucleus and the center of the egg at this time (Fig. 34) a mass of vesicular cytoplasm, derived from the inner sphere, entirely devoid of yolk-granules. As this substance preserves its individuality for a considerable time

and acts in a specific manner, it is best to designate it by a special name. So I shall speak of it as the *sphere substance*, following Conklin.

During the growth of the sphere substance the two germ-nuclei also have been enlarging. The individual chromosomes do not form separate vesicles, as in so many eggs, but are intimately united, apparently by processes sent out from the chromosomes. The two germ-nuclei begin their growth at the same time and keep pace with each other throughout the process; so that it would be impossible to distinguish them apart were it not for their positions and the relation of the egg-nucleus to the sphere substance.

The migration of the two germ-nuclei begins simultaneously some time before they have attained their full size. *The egg-nucleus is preceded in its first movements by the sphere substance, which stretches out towards the side of the egg opposite to the sperm-nucleus* (Pl. XXVI, Figs. 35 and 36); *at the same time the sphere substance undergoes a significant change of form, elongating in an equatorial plane at right angles to the line uniting the two germ-nuclei* (Pl. XXVI, Figs. 37 and 37 *a*).

The egg-nucleus thus moves at first away from the sperm-nucleus, which takes up its march in the direction of the former (Figs. 35 and 36). So far as my observations go, this is invariably the case. The two nuclei may meet a little to one side of the center of the egg, in which case they soon move to the center, or they may first come in contact at the center. *At the time of their meeting there is no indication of an aster in any part of the egg;* and I have only once found any such structure in the sphere substance; in this case the aster was exceedingly minute and no doubt sporadic in its appearance. In yet another egg I found an amphiaster in connection with the egg (?) nucleus some time before the meeting of the two nuclei. But this also was extremely unusual.

To return to the sphere substance: I have already spoken of its elongation in a horizontal plane at right angles to the line uniting the two germ-nuclei (Pl. XXVI, Fig. 37). *This elongation continues until there is a relatively narrow band of sphere substance stretching almost entirely across the egg* (Fig. 37 *a*), *at*

a time when the two germ-nuclei are still separated by a quarter of the diameter of the egg. The line of elongation of the sphere substance marks the long axis of the first cleavage spindle. Thus the first cleavage must pass very nearly through the point at which the sperm-nucleus has been resting.

The invariable migration of the sphere substance towards the side of the egg opposite to that in which the sperm-nucleus lies indicates one of two things: either that the sperm-nucleus has, so to speak, driven it away, or else that it is moving along lines of orientation of the egg-substance. The first alternative appears to me manifestly absurd, and the corollary of the second is that the sperm-nucleus has occupied throughout its entire resting period a definite position in the egg; and this can be explained only on the assumption of a definite orientation of the egg-substance, not only polar but also corresponding to the chief axes of the embryo.

It might very readily be assumed, on the other hand, that the plane of the first cleavage is determined by the copulation path of the germ-nuclei, as is stated to be the case in the ova of some other animals, frog (Roux, 87) and *Toxopneustes* (Wilson, '95). But whoever should take this position for *Unio* would have to explain how it happens that the sphere substance elongates in the plane of the future first cleavage spindle before the germ-nuclei come together. It would be necessary, I believe, to assume that the distant sperm-nucleus exercises an influence on the direction of elongation of the sphere substance in the first cleavage, although in the next division the sphere substance acts independently. And this assumption is absurd on the face of it. (For discussion of this, see p. 262.)

In the movements of the germ-nuclei there are two factors concerned: first, certain dynamic relations between the egg-substance and germ-nuclei, under which we include the influence exerted by the orientation of the egg-protoplasm; and, second, the mutual attraction of the germ-nuclei. The latter controls the course of the copulation path of the germ-nuclei to a great extent, but not altogether. It is generally, however, so strong a force that the influence of the former factor is not very apparent during the copulation path, but, after the germ-nuclei

have met, it is again in evidence in determining subsequent movements to the place of origin of the cleavage spindle, and later in determining the ultimate position of the latter. I am convinced, from its subsequent behavior, that the penetration path of the sperm-head is conditioned by dynamic relations with the egg-substance dependent on the orientation of the latter. "The paths of the germ-nuclei are determined by at least two different factors, one of which is an attraction or other dynamical relation between the nuclei and the cytoplasm, the other an attraction between the nuclei. The former determines the entrance path of the sperm-nucleus, while both factors probably operate in the determination of the copulation path, along which it travels to meet the egg-nucleus. The real nature of neither factor is known" (Wilson, '96, pp. 151 and 152).

Origin of the Cleavage Centrosomes.

When the two germ-nuclei come together there is no indication of an aster in any part of the egg. But, after they have been in contact for a short time, two centrosomes arise quite near together, one in contact with the egg-nucleus and the other with the sperm-nucleus, but both close to the plane of contact of the two (Pl. XXVI, Fig. 38. See description of this figure). I feel quite sure that the two centrosomes do not arise from the division of one immediately preceding, but are formed independently; for there is no trace of a central spindle between them, and at this stage not more than two rows of cytoplasm alveoli are involved in the rudimentary asters. All of my efforts to find in earlier stages any indication of origin from a single centrosome, either in closer approximation of the two, or in union by a central spindle, have been in vain, in spite of a superabundance of good material covering the critical period.

I was under the impression at the time of my first paper on this subject (*Science*, March, 1897) that the cleavage centrosomes arose in the sphere substance, and thought it possible, therefore, that they might be traced back to the egg-centrosome of the maturation divisions. But, although the centrosomes do arise very close to or on the margin of the sphere

substance (see Pl. XXVI, Fig. 38), I no longer believe that they are descendants of the egg-centrosome. Still less can they be derived from the sperm-amphiaster. *I believe that they are egg-products of new origin formed under the influence of the two germ-nuclei. It seems to me probable that each nucleus has the power in a certain condition of maturity to enter into a reaction with the cytoplasm, which results in the formation of an aster with its centrosome, and thus initiates the process of karyokinesis.* For instance, it sometimes happens in the egg of *Unio* that after the first cleavage the chromosomal vesicles unite so as to form two separate nuclei in each cell, instead of a single one. After these two nuclei have moved apart an amphiaster is formed in connection with each, and two karyokinetic spindles arise.

However, I have found no evidence in favor of the view of Carnoy et Le Brun ('97) as to *Ascaris*, that the cleavage centrosomes are directly derived from the nucleoli of the germ-nuclei. This view would be reduced to absurdity in the case of the egg of *Unio*, where in cleavage stages each chromosomal vesicle apparently forms a nucleolus.

The further formation of the first cleavage spindle is illustrated in Pl. XXVI, Figs. 39–43. The two centrosomes move to opposite ends of the plane of contact of the germ-nuclei. *Each subdivides several times to form a group of granules, which represents the rudiment of the inner sphere.* The outer sphere is well marked by the absence of transverse anastomoses of the rays. One of the centrosome granules then forms the center of the inner sphere, and the remainder arrange themselves around the periphery (Figs. 39–42). The rays of the aster form by continuity of neighboring alveolar walls, as is shown in the series of figures, and the central spindle is formed from the small amount of cytoplasm between the germ-nuclei on the one hand and the two centrosomes on the other.

Behavior of the Chromatin in the Germ-Nuclei.

During the growth of the germ-nuclei there is an enormous increase in the quantity of the chromatin (Pl. XXVI, Figs. 34–40), but a small proportion of which is used in the

formation of the chromosomes of the first cleavage spindle (Pl. XXVI, Figs. 41–43). What becomes of the chromatin which does not pass into the chromosomes? If the basichromatin granules, which stain so intensely in haematoxylin, were thrown out of the nucleus as such, it would be an easy matter to follow them, but apparently they are first transformed into oxychromatin, which does not stain in haematoxylin, and in this condition they are found on the mantle-fibers of the spindle, which differentiate out of the intranuclear network (Pl. XXVI, Fig. 42). However, some of the unaltered basichromatin is found near the spheres (Pl. XXVI, Figs. 42 and 43). A great part of the chromatin, then, is probably transformed into spindle fibers, as Wilson has maintained in the case of the sea-urchin; and a smaller part probably enters into close relations with the sphere substance, which it may influence through transfusion or other mode of transference.

The germ-nuclei never "fuse" to form a "segmentation nucleus," but from each arises a group of chromosomes. The chromosomes are at first long and narrow, and longitudinally split from the time of their first appearance (Pl. XXVI, Figs. 42 and 43). Later they shorten and condense, staining more intensely, and become short, bent rods (Pl. XXVI, Fig. 44, and Pl. XXVII, Fig. 45).

2. *Literature and Theory of Fertilization.*

It is well known that, in the majority of animals in which the process of fertilization has been studied with sufficient care, the cleavage centers arise from the sperm-amphiaster. (See Boveri, '87, '95; Crampton;[1] v. Erlanger, '97, '98; Fick, '93; Griffin, '96, '99; Hill, '95; Korschelt, '96; Kostanecki, '96; Mead, '95, '98; Sobotta, '97; Van Der Stricht, '97; Wilson and Mathews, '95.) When I began the study of the egg of *Unio* I had no reason to anticipate any other result than this, and had

[1] Crampton's work on the fertilization of the egg of *Molgula* (an Ascidian) is not yet published, but, as it has been ready for some time, the author kindly permits me to refer to it. This egg offers especially clear evidence of the persistence of the sperm-centrosomes in the cleavage, because there are no asters associated with the maturation spindles, and the sperm-asters are perfectly distinct, and persistent from the time of entrance of the spermatozoön.

certainly no prejudice against Boveri's theory. The reasons why it is impossible to take this view of the origin of the cleavage centers in *Unio* are: 1. The sperm-amphiaster disappears utterly before the metaphase of the first maturation spindle, and the sperm-centrosomes become indistinguishable from cytomicrosomes. 2. There is no evidence of the persistence of the sperm-centrosomes during the maturation as anything more than cytomicrosomes, unless we identify with them the centrosomes of the accessory aster, which, however, contains at first only a single centrosome. 3. The cleavage centers *always* arise in a definite position, *viz.*, on each side of the plane separating the two germ-nuclei, one in contact with each nucleus.

If the cleavage centrosomes really are the persistent sperm-centers, it becomes necessary to assume: 1. That the sperm-centrosomes, which are no larger than cytomicrosomes, maintain a persistent identity among these, though the chief distinguishing mark of a centrosome is its position in the center of an aster or centroplasm. 2. That one of the sperm-centrosomes becomes active during the second maturation division (accessory amphiaster) and divides again, the resulting amphiaster then vanishing. 3. That two of the three sperm-centrosomes migrate *to the perfectly definite position*, in which the cleavage centers arise, and there again resume activity. If any theory of fertilization, *otherwise tenable*, depended on such assumptions, I should feel justified in making them. But it seems to me that Boveri's justly celebrated theory must be given up, in large part at least, for other reasons to be spoken of below.

As already mentioned, disappearance of the sperm-centrosomes has been observed by a number of authors (pp. 233, 234). Of these authors, *viz.*, Child, Van Name, Foot, MacFarland, and Klinckowström, only MacFarland positively maintains that the cleavage centers arise from persistent sperm-centers, and his argument appears to me most inadequate. The only reason that he gives for his positive identification is, that the cleavage centers may arise in almost any position relative to one another and the germ-nuclei, though always well above the vegetative pole; and the sperm-centrosomes exhibit a similar relationship,

or lack of relationship to anything definite, before their disappearance. The case of *Arenicola* (Child, '98) gives especially clear evidence of independent origin of the cleavage centers, for in this case the sperm-centrosomes do not disappear until after the formation of the second polar body, and the cleavage centrosomes arise soon after in such a position that it seems simply impossible that they should represent persistent sperm-centrosomes. Child himself is "inclined to regard the cleavage centrosomes as new formations and as not related to the 'male' centrosome." In the other cases there is certainly no objective evidence of identity of sperm-centrosomes and cleavage centrosomes, indeed much that speaks against it.

I agree, therefore, with Wheeler and with the views expressed by Korschelt (95b, p. 655) and Brauer ('93) that the centrosomes are of no special significance in fertilization. The cleavage centers may arise from the sperm-centers, or from the egg-center (doubtfully, *Myzostoma*,[1] Wheeler, '97), or in

[1] I cannot regard Kostanecki's observations on *Myzostoma* ('98) as *proving* the origin of the cleavage centers from the sperm-centrosomes in this case. According to both his observations and Wheeler's ('97) no sperm-aster is visible (until at least after the formation of the second polar body, Kostanecki), although the spermatozoön enters the egg before the breaking down of the germinal vesicle. The sperm-nucleus always lies toward the vegetative pole. The germ-nuclei enlarge simultaneously, and begin to approach, the sperm-nucleus making a more extensive migration than the egg-nucleus, so that their place of meeting is always nearer the animal pole. When the nuclei have approached within a short distance of each other, there appears between them a usually double aster (source of cleavage centrosomes). Sobotta's observations on the mouse are entirely similar.

What is the origin of these centrosomes? The author (Kostanecki) sees only two possibilities: either they are the egg-centrosomes reappearing, or the sperm-centrosomes.

"Ich habe aber überdies *vereinzelte* Bilder bei Myzostoma gesehen, die direct für die Herkunft der Centrosomen von Spermatozoon sprechen: wenn die beiden Geschlechtskerne die Gestalt von grösseren Bläschen angenommen haben, konnte ich an der Eimitte zugekehrten Seite des Spermakerns *bisweilen die Andeutung* einer Strahlung sehen, die jedoch mehr durch eine radiäre Anordung der kleinen Dotterkörnchen zum Ausdruck kam. Die radiär angeordneten Körnchen umgaben ein helleres Feld, in dessen Mitte ein oder zwei kleine Punkte zu sehen waren. . . . Ich glaube in allen diesen Fällen (Pl. XXIV, Fig. 14; Pl. XXV, Figs. 15, 16) das Spermacentrosoma oder die Spermacentrosomen vor Augen gehabt zu haben, wenn auch in Anbetracht des Umstandes, dass das Hauptkriterium für die Existenz von Centrosomen, nämlich eine *deutliche* Strahlung, hier fehlte, eventuelle Zweifel nicht mit absoluter Sicherheit zurückgewiesen werden können."

But these "doubtful" sperm-centrosomes were never traced to the cleavage

such a way that there is no discernible relation to either (*Unio, Arenicola, Allolobophora, Pleurophyllidia, Planocera, Myzostoma, Mus*). (For the last case see Sobotta, '95.)

It must certainly be admitted that the most recent observations on the fertilization and early cleavage of the ovum demonstrate the inadequacy of Boveri's theory of fertilization, *viz.*: "The ripe egg possesses all of the organs and qualities necessary for division excepting the centrosome, by which division is initiated. The spermatozoön, on the other hand, is provided with a centrosome, but lacks the substance in which this organ of division may exert its activity. Through the union of the two cells in fertilization all the essential organs necessary for division are brought together; the egg now contains a centrosome, which by its own division leads the way in the embryonic development." (Quoted from Wilson, '96.) Certain observations, such as those of Wilson, Mead, Griffin, and others, showing that the sperm-centrosomes become the cleavage centers, support it. But in other cases it is not possible to trace any connection between the centers of the first cleavage and the sperm-centrosomes. And it has been shown by Morgan ('99) that certain chemical substances may initiate cleavage of the unfertilized egg by stimulating the egg-cytoplasm. Loeb ('99) has recently shown by an experiment, whose conception and brilliant success must win our warmest admiration, that in the egg of *Arbacia* not only may division be initiated by adding certain chemical substances to the sea water, but that the entire typical development may take place under these circumstances without fertilization. Neither the sperm-centrosome nor nucleus is required for the typical development of the sea-urchin egg, which has long served, on account of the clear way in which the sperm-centers may be shown to become the cleavage centers in normal development, as the paradigm of Boveri's theory. Certainly in this case the egg is not without the mechanism of cell division, though this mechanism may usually be inhibited by the more active development of the sperm-centers. If not here,

centrosomes. These observations can thus be convincing evidence of the spermatic origin of the latter only for those whose minds are made up in advance.

then nowhere, can the theory hold. Moreover, it seems to me that the theory loses all its significance if the view that the centrosome is a unique and persistent organ of the cell should be given up, as now seems necessary, since it has been shown that centrosomes may arise at any place within the cell, by the observations of Mead ('97), Morgan ('96 and '99), Reinke ('94), and Watasé ('94).

Fertilization usually accomplishes two purposes: 1. It sets in motion the series of developmental phenomena, the entire mechanism for which is in the egg, awaiting only the proper occasion or stimulus. This is not effected primarily by the sperm-centrosomes furnishing cleavage centers, as shown above, but probably by some stimulus, chemical or mechanical, emanating from the part of the spermatozoön that penetrates.[1] 2. Fertilization restores the typical number of the chromosomes. In this we must recognize its chief function and so return to earlier theories of fertilization. The intercalation of a sexual generation in the series of asexual or parthenogenetic generations, wherever these may occur alike in plants and animals, demonstrates, as has been so often pointed out, the fundamental significance of amphimixis. We may regard the first function of fertilization, that of setting in motion the series of ontogenetic changes, as an accessory one adapted to secure the fertilization of as many eggs as possible and to prevent recurrent parthenogenesis. This is of course but a modification of Boveri's theory, that the *postulated degeneration* of the egg-centrosome is an adaptation against parthenogenesis. Perhaps Mead's suggestion, "The inhibition of division would seem to depend upon the metabolic activity peculiar to the cell by virtue of its internal structure," may partly explain the mechanism of this adaptation of the egg-cell (Mead, '98b, p. 218). Or Loeb's view: "The ions and not the nucleus in the spermatozoön are essential to the process of fertilization" ('99, p. 137).

[1] The universal, or almost universal, occurrence of sperm-asters might thus be interpreted simply as the first response of the egg-cytoplasm to this stimulus.

IV. Movements of the First Cleavage Spindle; First and Second Cleavages.

The first cleavage spindle is invariably formed in the exact center of the egg, as already described, and parallel to the plane of elongation of the sphere substance, which stretches completely across the egg. The spindle remains in the center of the egg until just before the metaphase, and then, without undergoing any considerable change in form or size, it moves end first towards one side of the egg, until one sphere comes almost in contact with the cortical alveoli (Pl. XXVII, Figs. 45 and 46). This movement is a necessary preparation for the first cleavage, which is very unequal. But it is difficult to understand why the spindle should first form in the center of the egg and secondarily change its position. The metaphase and almost the entire anaphase (Pl. XXVII, Fig. 47) of the spindle are spent in this position, and then the spindle shifts back a little (Pl. XXVII, Figs. 48 and 49) to the definitive position of the cleavage plane. (See descriptions of these figures.) It is possible that there may be other lesser oscillations of the spindle before the point of equilibrium is found.

It is perfectly plain from this description that the position of the spindle does not primarily determine the place or direction of the first cleavage plane, but that, on the contrary, the position of the spindle is controlled through the cytoplasm, as a needle in a magnetic field oscillates until equilibrium is attained. Now there are no visible differences in structure in different parts of the egg, which might explain the movements of the spindle, but it seems to me that the movements themselves are an indication of a definite bilateral orientation ; for I cannot conceive it as being possible that in an isotropic substance movements of such definiteness and prospective value in the cleavage should take place. Thus, although there is no other evidence that the movement of the spindle in determining the inequality of the first cleavage is in one direction rather than the other, I am convinced that it is always in the same direction under the influence of some predelineated organization of the cytoplasm.

The behavior of the spheres in the first cleavage is entirely similar to that of the egg-sphere during the copulation phases of the germ-nuclei. The enlargement of the inner spheres begins during the anaphase (Pl. XXVII, Fig. 47) and goes on rapidly during the telophase, until a large amount of sphere substance is produced (Pl. XXVII, Figs. 48 and 49). The sphere substance then elongates in the direction which the second cleavage spindles will assume much later (Pl. XXVII, Fig. 50, horizontal section, and Figs. 51 and 52, vertical section, of the larger cell *CD*). *At the same time each cell takes on the form of a dividing cell* (seen especially well in the larger cell, Pl. XXVII, Figs. 50–52), *although the nuclei are not yet reconstructed since the preceding division.* I regard this as a premature expression of the forces within these cells that later cause an unequal division in the region of the constrictions, after the spindles have been formed. Later both cells round off, then become closely appressed, and, when the spindles form, they move into the positions previously indicated by the elongation and constriction of the cells and sphere substance.

The sphere substance is thus divided in both the first and second cleavages; from its position and form it seems likely that it is distributed between the cells very nearly in proportion to their size. It is, however, impossible to be certain of this, because one cannot follow it in any division up to the time of the definitive placing of the spindle; before this time the intrusion of yolk-granules on it has hidden it from view.

V. CONCERNING THE SPHERE SUBSTANCE.

From the preceding account it will be plain that I regard the entire sphere substance as the product of growth or inflation of a single centrosome. There can be no doubt that it is derived from the inner sphere, so that everything depends on the interpretation of this structure. On pp. 240–242 I have given in detail my reasons for believing that each of the inner spheres of the second maturation spindle is derived from a single centrosome. I can add nothing to the description there given, except the statement that I have worked over this series of

phenomena repeatedly with the utmost care, and have demonstrated the sections to several colleagues with perfect satisfaction. The inner sphere at the central end of the spindle becomes the sphere substance of the fertilized egg. The evidence that the sphere substance formed at the poles of the first cleavage spindle has a similar centrosomic origin is equally conclusive, as the figures show. It seems very probable, although I cannot speak with assurance on this point, that part of the material for the growth of the sphere substance is derived from the nucleus. If this be so, the later wide dispersal of this substance through the cell may give a useful clue to the manner in which the nucleus exercises its undoubted formative influence on the cell.

A similar growth of the spheres towards the end of karyokinesis is now known to be of very wide occurrence (Agassiz and Whitman, '89; Boveri, '95; Erlanger, '98; Herfort, '99; Conklin, '99; Vejdovský, '88). Opinions as to the morphological significance of these structures vary. Erlanger ('98) declares: "Ferner entsprechen die Centroplasmen oder 'Sphären' durchaus nicht riesig angeschwollenen Centralkörpern wie Boveri angiebt." My own observations would support Boveri's interpretation. Erlanger also suggests an interaction between nucleus and sphere substance: "Den hier entwickelten Anschauungen gemäss dürfte in den mittleren Phasen der Mitose ein enges Verhältniss zwischen den Centroplasmen, inclusive Centralkörpern, und dem Kern, beziehungsweise Kernspindel, herrschen." Herfort's observations ('99) lend themselves to a similar interpretation.

Conklin ('98) was the first to recognize the possible importance of the sphere substance in differentiation. He summarizes his results thus: In the egg of Crepidula, "after the first two cleavages, the sphere substance is differently distributed to the different cells, the entire sphere substance of one generation always going into those cells of the next generation which lie nearest the animal pole. This differential distribution of the spheres has been followed through every cleavage up to the 24-cell stage. As the form of cleavage is perfectly constant, it follows that the sphere substance of any generation

goes into certain definite cells which have a perfectly constant origin and destiny.

"This differential distribution of the spheres is not caused by their specific weight, since their movements are the same in whatever position the egg may be placed. It seems to be the result of a form of polarity, which, like that of the egg itself, is not the result of gravity.

"The centrosomes do not, apparently, arise from the sphere substance of the previous division, but some distance from it, and the sphere substance itself never divides, but each sphere ultimately grows ragged at its periphery, and gradually fades out into the general cytoplasm.

"The differential distribution of the spheres and their subsequent conversion into cytoplasm suggests that they may be important factors in the differentiation of cleavage cells, and if further investigation should establish the fact that they are in part composed of the oxychromatin of the nucleus, it would furnish a basis in fact for certain well-known speculations of de Vries, Weismann, and Roux."

VI. TWO RECENT THEORIES OF UNEQUAL CLEAVAGE.

Before proceeding to a general consideration of the organization of the egg of *Unio* let us take up two recent theories of unequal cell division. Those views that seek a simple mechanical explanation either in the arrangement of yolk or other inclusions, or in extrinsic forces, have been already sufficiently criticised by Jennings ('96), Conklin ('97), zur Strassen ('98), myself ('95 and '99), and others.

1. *Ziegler's Theory of the Unequal Power of the Centers.*

Ziegler has suggested the theory of centers of unequal power for the explanation of certain unequal cleavages in the ontogeny of nematodes and the ctenophores ('96 and '98). He styles an unequal cleavage in which the centers are of unequal power "heterodynamic." Ziegler does not claim, himself, to have observed any difference in the centers in such cases, but he

believes that in many cases of unequal cleavage there is no other explanation possible. The theory certainly is an advance over the blind faith in invisible yolk that plays so prominent a part in Hertwig's theorizing ('92 and '98) in such matters, inasmuch as it squarely meets the conditions of the problem — unequal cleavage in a mass of protoplasm, in which yolk may be absent or uniformly distributed. But it appears to me to be without satisfactory foundation in fact. If the postulated difference really exists, it should be evident from the start in unequal mastery of the cytoplasm by the two centers ; but if we take the case that Ziegler himself instances as the most marked example of "heterodynamic" cleavage, the formation of the polar globules, we find that in the first maturation spindle, when first formed and lying horizontally, there is no discoverable difference in the size or appearance of the centrosomes or asters at the ends of the spindle. It is only after the spindle has rotated, and one end has moved out to the surface, that a difference is to be noted; and this is a secondary difference, due not to unequal force but to unequal opportunity. The same is true of every unequal cleavage of the entire ontogeny. As Conklin has pointed out, the centrosomes accommodate themselves to the size of the mass of cytoplasm *in which they come to lie*, being at first equal. Inequality of centers and asters is an effect, not a cause, of unequal cleavage.

But one has a right to expect of a theory meant to be purely formal, that it should be at least far-reaching ; here, however, we are told simply, the cause lies not in the protoplasm but in the centrosomes. How the centrosomes become of unequal power, and how this is adapted to embryo formation, of this not the slightest clue.

It goes without saying, then, that I cannot find in this theory any explanation of the unequal cleavage under discussion. I have looked in vain, though with great care, for any difference in the two centers of the first cleavage, while the spindle still occupies the center of the egg. A difference becomes noticeable only after the spindle has become excentric, and this is plainly due to the fact that the centers are now operating in areas of unequal extent.

Child ('97) finds a difference in the size of the cleavage asters, and suggests that "this anticipates and indicates the difference in size of the first two cells of cleavage." The segmentation nucleus is shown in an excentric position in the figure illustrating this, and I would suggest that the difference in the asters may be due to this.

2. *Conklin* ('99) *ascribes unequal cleavage to movements of the cytoplasm:* "When the cell movements carry the mitotic spindle out of the middle of the cell, unequal cleavage results." The nature and cause of the movements are thus described: "With the escape of nuclear sap into the cell body at the beginning of mitosis, vortical movements are set up in the cytoplasm, the poles of the spindles being the centers of such vortices." The movements are thus "due to the appearance of unlike substances in different parts of the cell." The extremely delicate and beautiful observations on which Conklin's conclusions rest form a model of morphological methods. I fully believe that we have here a factor that must be reckoned with in our theories of early differentiation; it may even have all the importance that Conklin claims for it, while it certainly fails, like all its predecessors, to give any clue to the solution of the adaptive aspect of these phenomena. It may well be that the shiftings of the first cleavage spindle in *Unio* are due to currents in the cytoplasm; the changes in form of the sphere substance and the elongation of the cells after cleavage furnish possible evidence of such currents. But why these currents should relate themselves to subsequent events by placing the spindle in its definite position we cannot say. It would seem also to be necessary to distinguish carefully between random cytoplasmic streamings and those that are definitely adapted to purposes of differentiation. For instance, Ziegler ('96) describes how the germ-nuclei in the egg of *Diplogaster*, lying near opposite ends of the egg, are carried around at first in an aimless fashion by streaming movements of the cytoplasm, which do not, however, prevent the union of the germ-nuclei near the end of the egg, destined to become the posterior end of the embryo. The spindle is then formed and rotates so as to lie in the long axis of the egg, frequently oscillating for a

time back and forth across the line of equilibrium. Even after this the spindle may be carried around more or less by streaming of the protoplasm, and yet it comes to rest for the division in a definite place; but it *may* be that the random streaming has become converted into a slow, directive movement that determines the position of the spindle. This *must* be the case, if the theory is to apply here. The theory would seem to imply that protoplasmic currents may be adapted to purposes of differentiation, not that all streaming is definitely directed; just as cell division may be adapted to purposes of differentiation, though it may equally be without definite reference to it.

VII. Organization of the Egg.

We have been assured frequently of late by Driesch (*e.g.*, '99, p. 717) that descriptive investigations can at most yield negative results, that only experimental results justify general theorizing. While I would personally agree with Driesch that such theorizing as he sometimes indulges in cries aloud for justification, I do not find anything in his remarks to cause descriptive writers the slightest qualms in drawing conclusions from their observations. "Negative" and "positive" are purely relative terms, and as the scientific method at present universally followed consists in testing working hypotheses by all means available, I cannot see why a fact of pure observation should be "negative" in a different sense from one based on experiment; or less instructive, especially in biology, where most of the conditions are unknown, and the experimenter is generally unaware of much that he has modified. I should be the last one to deny the value of the results reached in the field of experimental embryology, in which Driesch has done such brilliant work; but surely it is time to object when this writer claims monopoly of the faculty of judgment for those who have shaken eggs. Rather in our speculation should we check our observations with experiment and our experiments with further observation, for only one thing is more deadly than a careless observation, and that is a doubtful experiment, and either may lead astray. Surely one who has

so often been led astray, on his own admission, by doubtful experiments should realize that everything lies in the interpretation.

One would suppose that even Driesch would admit that the problem of the development of an entire egg is no more complicated than that of a part; and that the fact that a part may develop into a normal embryo gives no explanation of how a normal embryo is formed, either in "absolut-normal" or in modified cases.

The evidence on which I base my argument for a bilateral, and more than bilateral, organization of the cytoplasm of the egg of *Unio* may be summarized thus: 1. The polar differentiation is clear and definite, but without reference to the distribution of yolk. 2. Evidence of a *bilateral* orientation of the protoplasm is given by the behavior of the germ-nuclei and of the sphere substance; the line uniting the two germ-nuclei just before they come together is at right angles to the long axis of the future first cleavage spindle, and the plane of elongation of the sphere substance is parallel to the future axis of this spindle. We have, then, two independent phenomena that have definite reference to the future axes of the embryo, for the first cleavage spindle has such a definite relation. In the absence of definite elongation of the sphere substance it would be possible to interpret the determination of the axes of the embryo as due to the plane of meeting or of copulation of the germ-nuclei. The coincidence of two such independent phenomena can only be interpreted as due to a third factor, *viz.*: bilateral orientation of the egg-cytoplasm. 3. Evidence of differentiation of anterior-posterior proportions is found in the shifting of the first cleavage spindle. We have seen that this shifting can be due neither to unequal distribution of the yolk nor to unequal power of the cleavage centers. Moreover, the final position of the spindle has a differential value in the subsequent ontogeny, *i.e.*, it is adapted to the formation of a large shell gland and mesodermal structures, as I showed in my paper on "Adaptation in Cleavage."

In this paper I pointed out that the only possible basis for a rational interpretation of the rate, direction, and place of

cleavage in the cells of the egg of *Unio*, and of other animals possessing strictly determinate cleavage, is a prospective one. "In the cleavage of the egg of *Unio* there are marked variations in the size of the cells and in the rate and direction of their cleavages; in every case these possess prospective significance, and by means of them the organism is able, so to speak, to realize, in the most direct manner possible, on its available capital, the substance of the egg. *To this principle I have given the name of adaptation in cleavage*" (*l.c.*, p. 53). I must refer to this paper and to an earlier one, "Embryology of the *Unionidae*" ('95), for the details of proof that there is no relation between the form of the cleavage and the distribution of the yolk or any extrinsic forces.

The larger of the first two cells is destined to form the posterior part of the embryo, including the mesoblast and the immense shell gland. The smaller cell will form the relatively small anterior portion of the embryo. The proportions of these parts are, then, marked out by the first cleavage. The place of the first cleavage is due to the position ultimately occupied by the first cleavage spindle. The manner in which this reaches its excentric position has already been described in detail. It is certainly due to inherent forces of the undivided protoplasm. Before the egg of *Unio* has divided for the first time it thus foreshadows not only the polarity (position of ectoderm and endoderm), but also the position and *proportions* of anterior and posterior parts of the embryo. *That is to say, the forces within the unsegmented egg of Unio are those not only of a bilaterally symmetrical animal, but of such an animal of definite proportions.* This much at least is evident; how far other features of the embryo are actually existent in the unsegmented egg, and how far they arise in response to subsequent conditions, internal and external, I am unable to determine. I simply repeat that, independently of cleavage or other of the phenomena usually styled developmental, the bilaterality and the proportions of the embryo of *Unio* are dependent on a certain distribution of protoplasmic (as opposed to nuclear) forces. Nor are these forces in any way complicated or modified by subsequent events; rather is

the course of subsequent events guided by their unbroken continuance.

These forces are responsible in part for the paths of the germ-nuclei, the elongation of the sphere substance, the definite position and shifting of the spindle, and later, to a great extent, for the equality or inequality, the rate, and the direction of the early cleavages.

The observations and experiments that force on us the conclusion that the cytoplasm of the egg is definitely organized are now so numerous that a book would be required for their adequate consideration. I will therefore refer to only a few typical examples. Watasé ('90) shows that the unsegmented blastoderm of the squid (*Loligo*) is bilateral in structure, the nucleus being situated excentrically nearer the end of the blastoderm that is to become the posterior end of the embryo. The blastoderm is at the pointed end of the nearly oval egg, which is more convex on the surface corresponding to the anterior end of the blastoderm. Watasé does not hesitate to attribute the bilaterality of the blastoderm to the distribution of different protoplasmic substances. He cites the following interesting extract from van Beneden and Neyt on *Ascaris* (1887): "Les premiers blastomères ont, comme l'œuf fécondé, non seulement une symétrie monoaxone, mais une structure bilatérale. Il est probable que c'est là un caractère commun à toute cellule et l'on doit concevoir un organisme cellulaire, non comme formé de couches concentriques, mais comme présentant essentiellement un axe à extrémités différents et un plan unique de symétrie. Cette symétrie bilatérale de la cellule est probablement la cause de la symétrie bilatérale des organismes plus complexes, des animaux en particulier."

McMurrich's observations ('95a, '95b) furnish interesting evidence of a high grade of organization of the ova of Isopoda. In *Jaera* the segmented egg is a syncitium, the cells being really local nucleated accumulations of a continuous cytoplasm in which the yolk is imbedded. The egg is oval, so that it is possible to recognize an anterior (broader) and posterior (narrower) end. In the 8-celled stage the arrangement of the cells is as follows: four cells at about equal distances apart surrounding

the anterior end of the ovum, near the posterior pole a second circle of three, "while the eighth cell *D* occupies an almost polar position." In the undivided egg there is a central nucleated mass of protoplasm connected by strands with a peripheral layer. Divisions begin in this central mass and do not affect the yolk, so the cells are united by protoplasm, although they move apart. This lack of contact of the cells characterizes the early cleavage throughout, so that, as McMurrich points out, none of the laws of alternating cleavages or mutual pressure can influence the form of cleavage. Yet the cleavage does follow an invariable law of its own, and the arrangement described for the 8-cell stage is brought about partly by the orientation of the spindles and partly by extensive migrations of cells. Now it is to be observed that this arrangement is an adaptive one in the sense that it predelineates definite systems of organs. The four anterior cells give rise to the ectoderm, the three next to entoderm and some mesoderm, while the posterior cell forms the vitillophags, "still later giving rise to certain mesodermal structures."

"In the later stages of *Jaera* there is a concentration of cells towards one surface of the ovum, which will eventually become the ventral surface of the embryo; concomitantly with this concentration the outlines of the naupliar region of the embryo being formed." But in *Porcellio*, "at the period at which the concentration of the peripheral protoplasm occurs," to form the ventral region of the embryo, "the nuclei are separated from it by equal and considerable distances, being united with it, however, by the protoplasmic network, and it is difficult to conceive how any of them could be able to influence the peripheral cytoplasm in such a way as to produce the concentration. It seems rather that we have to do with an independent action of the cytoplasm, which precociously prepares for the formation of the blastoderm."

Have we not here a beautiful picture of the way in which the egg acts as an undivided organism, controlling some events by orientation of cleavage planes or shifting of cells, and others by protoplasmic movements clearly independent of cell boundaries? Is it not evident that the orientation of the cytoplasm

is the primary factor in these cases? and that this organization must include at least bilateral symmetry and possibly definite proportions?

In his "Inadequacy of the Cell Theory of Development," Whitman has given a picture of the way in which crystallization of the embryo sets in in the undifferentiated mass of cells composing the blastodisc of the pelagic fish-egg. "It is well known that the transformation of the blastodisc just before the appearance of the germ-ring is quite rapid, at least in the pelagic fish-egg, and also *quite independent of cell formation.* The discoidal germ-mass suddenly thins out, but not uniformly in all parts. The half of the disc in which the embryo is to be formed remains thick, anticipating, as it were, the axial concentration which is to follow, while the half lying in front of this is rapidly reduced to a thin epithelial membrane. This *regional* differentiation of the outer layer and the concomitant formation of the germ-ring, including the forward movement of the embryonic plate ('head process'), which advances in an axial direction to the very center of the disc, are indubitably accomplished, not by the aid of cell formation, but by formative processes of an unknown nature, but, nevertheless, real and all-controlling. Cell formation, to be sure, goes on, but it seems to me certain that it has no *directive* influence on the formative process. The cleavage runs on from beginning to end, regularly or irregularly, without modifying in any essential way the form of the blastodisc. All at once, when this segmentation has been carried to a certain point, the transformation sets in and goes rapidly on, without interrupting cell formation, but to all appearance quite independently of it."

The view that the cytoplasm of the egg possesses at least a definite bilateral orientation is not in the least weakened by cases of non-determinate cleavage. In such cases all one can say is, that cell formation is not directly adapted to purposes of differentiation. But is this view disproved by such observations as those of Wilson ('95) and Ziegler ('96) that the orientation of the embryo may bear no definite relation to the polarity of the egg? We must inquire here what is meant by this expression, *polarity of the egg.* Two things, clearly, are meant

by this, and it is necessary to sharply separate these in our thinking, if not in our vocabulary. In the first place is meant the distinction that is marked by the appearance of the "polar" globule at a point (*i.e.*, "pole") on the surface; in the second place is meant the distinction that is based on the existence of ectodermal and entodermal portions of the blastula or gastrula. Now formation of polar globules is historically a very different thing from embryo formation, and there is no other reason for assuming that the axes marked out by these distinct processes *must* correspond, than that apparently they almost always do. Therefore I fail to see why such observations as those of Wilson and Ziegler furnish evidence against such an organization of the egg-cytoplasm as we have maintained. If there be an ectental orientation of the egg-cytoplasm prior to maturation, it would be natural to expect a control of the place of polar globule formation by this, of such a sort that the polar globules would tend to arise at the ectodermal (animal) pole, as is usually the case, or entodermal (vegetative) pole, as happens apparently in the Ascidian egg (Castle). But one can readily imagine that other forces might operate more powerfully and determine a different location of the polar globules.

Just as little do I consider that observations of the development of the egg under pressure or of the development of parts into whole embryos militate against the view here upheld. The eggs, like the adults of many animals, possess great power of reorganization. In such cases the reorganization of the egg-cytoplasm would be a necessary preliminary to typical development, however we may think of it as taking place.

Finally, even if it should be proved that the axis of the embryo may be determined either by the point of entry or by the copulation path of the spermatozoön, we should have here no evidence of lack of previous organization in the cytoplasm, but simply another striking confirmation of the wonderful power of reorganization of the egg-cytoplasm, for it has been shown that parts of fertilized eggs in which this process is held to occur (frog, sea-urchin) are capable of typical development, in which case the path of the spermatozoön can exercise no possible directive influence.

If the basis of these adaptive cleavage forms is cytoplasmic, as I believe, the complexity of the cytoplasmic organization must be great. The preceding examples illustrate this. But no phenomena of this sort give so overwhelming a sense of the inadequacy of our theories of development as the fact that cleavage may actually exhibit ancestral reminiscence (Wilson, '98). Admitting that reminiscence of ancestral conditions is ever shown in ontogeny, no one who is conversant with the literature of cell lineage can doubt that Dr. Wilson has placed the phenomena which he describes in their natural category; they belong to the same order of events as the development of tooth germs in a whale embryo. No one who has become convinced that true homologies are found in cleavage, and that the cleavage may exhibit adaptive modification, can be surprised by this last discovery. But these phenomena bring us so near to the unsegmented egg, that the conclusion is unavoidable that homology, adaptation, and ancestral reminiscence have a protoplasmic basis, and are not merely dependent on the recurrence of similar conditions.

Another conclusion that follows directly from this is that the egg transmits far more than the spermatozoön, *at least in these early stages.* All those features of the early development that are dependent on the organization of the cytoplasm must be transmitted by the egg alone. Driesch ('98) has gone to work in the right way with his experiments on hybridization, to determine what features of the early development are purely maternal in their origin. Before his experiments were published I had begun some similar experiments on fish-eggs to determine the influence of hybridization on the cleavage; but reached no certain results, owing to difficulties offered by the material. Experiments of this sort on eggs exhibiting determinate cleavage are much to be desired. The annelids would offer, perhaps, the best opportunities for this kind of work.

Coördinated and definitely directed forces imply a mechanism of definite configuration; what idea can we form of the configuration of this mechanism? In the first place, it should be observed that, inasmuch as the capacity for typical development is not dependent on the maintenance of the integrity of

the entire egg, but is, as has been shown for echinoderms especially, the property of any nucleated part of sufficient size, the mechanism cannot be *primarily* due to structurally differentiated germ-areas. Even if such areas can be shown to exist in any unsegmented egg, it would be necessary to regard this as a secondary effect of the primary mechanism upon the unsegmented egg.

It has not been shown for the egg of *Unio* that separated nucleated parts of definite size are capable of typical development; but even if it should be shown that they are not, that typical development is dependent on the integrity of the original egg, I could not consider that as evidence in favor of localized germ-areas; for it has been clearly demonstrated, in the case of the frog at least, that partial development of isolated blastomeres may be due to certain physical properties of the protoplasm. Whether we are studying the typical development of a part of an egg or of the entire egg, we are confronted with the same problem; and all that the facts of typical development of a part teach us is, that neither the nuclei nor yet the protoplasm of early cleavage stages are unchangeably specified. Are these then the "positive results" that we are led by Driesch to expect? So far as positive results are concerned the entire problem still faces us, and the negative conclusions were clearly seen by some naturalists as the result of interpretation of facts of observation ("Nature's Experiments"), long before the experiments were performed.

But we may be told that complete and typical development of a part, even from the moment of its separation, is no argument against the original localization of germinal areas, for the typical regeneration of a fragment of *Hydra* does not alter the fact that the parent animal had differentiated parts. But in the absence of ocular demonstration of areas in the whole egg, or of experimental proof of their occurrence, and in the face of the fact that currents of rotation and translocation in the protoplasm of the egg in no wise affect the power of typical development (Ziegler, '96), the theoretical necessities must be hard that will drive us to such an hypothesis. Moreover, if certain areas be removed from the egg, for instance, *A* and *B*

from an egg *A B C D*, then, according to the hypothesis, *C* and *D* must reconstitute *A* and *B* before the typical development can begin. But it can be shown that they are not reconstituted by growth; and *if complete reorganization of the areas take place in the material of the part, their typical distribution and proportions must be due to some primary mechanism of the entire protoplasm independent of regional differentiation.* Finally, in the regeneration of *Hydra*, the fact that equal parts from different regions form similar individuals shows that the power of regeneration does not depend upon the visible structural features, but on some underlying principle of organization common to all parts of sufficient size. To argue, therefore, for germinal localization from the facts of regeneration is to miss the most characteristic feature in such regeneration as that of *Hydra*, for the visible localization of parts has no reference to the formative forces.

Driesch has also been concerned with the problem of the configuration of the protoplasmic mechanism in the egg. In his paper "Lokalisation morphogenetischer Vorgänge" he formally announces his discovery that the location of morphogenic phenomena is a problem *sui generis*. After devoting a considerable part of the introduction to the demonstration of this, he begins Part I thus: "Having shown that the localization of ontogenetic processes is a possible problem, we begin by instancing a further example of this problem by recording the results obtained on blastomeres of the echinoderm egg." Does Driesch really believe that all the rest of the world has remained in ignorance of the existence of such a problem? Is he not aware that the theory of localized germ-areas is shaped largely to the requirements of this problem? What does he suppose that Loeb has in mind when he propounds the question, "What are the circumstances which determine that only one kind of organs originate at *certain places* in the body?" ("On Some Facts and Principles of Physiological Morphology," *Biological Lectures*, 1893, p. 38). It has long been generally recognized that the morphological problems of any ontogenetic process involve at least three factors: (1) its localization; (2) the time of its origin; (3) its specific nature: (place, time, structure).

I dwell on this because I am especially concerned here with a problem of localization, *viz.*: What determines the position of the first cleavage plane? and this, as I have attempted to show, is equivalent to asking, What determines the general proportions of the embryo of *Unio?* With Driesch I recognize that the conditions of this problem are found in a mass of protoplasm, the parts of which cannot be shown to be different.

Now how does Driesch conceive of this mechanism? In the first place, he attempts to explain the polarity of the egg by assuming polarity of the protoplasmic parts or elements, without considering further the nature of these; and as he cannot find the causes of the bilateral structure of the embryo in external conditions, he endows the parts with bilaterality also, so that each is doubly heteropolar. The problem of the polarity and bilaterality of the egg is thus transferred to its parts, so that the organization of the whole might be pictured as due to a "magnetization of the parts." But these postulates are still inadequate to explain the localization of ontogenetic processes; why the stomodaeum, for instance, arises in a certain part, or why the intestine of the pluteus becomes divided into three parts of definite proportions. How is this condition satisfied in Driesch's system?

"Let us first examine the simpler case of differentiation in a system with but one heteropolar axis, for instance, the intestine of the echinid gastrula, or the stem of *Tubularia*. Here we can represent the process in 'causal' form to ourselves by assuming that one of the end points of the axis is the seat of incitive (auslösender) 'Fernkräfte'; this spot, which is distinguished as something different from other points of the system by the very fact that it is the end of the axis, produces, therefore, an effect (*Wirkt* also); that on which the effect is produced must be able to answer to the cause, as in all cases where effects are produced; and in this manner a kind of causal harmony is postulated by this conception of the matter.

"In what way, now, does this particular designated place of the axis affect the system? Let us explain this first in the

simple case of the segmentation of the intestine of the echinid gastrula.

"Here at first only two effects are produced; the formation of two constrictions takes place. That the effect happens to be the formation of constrictions does not concern us here, for it is founded on the potency of the system, and is only called forth by the exciting cause; but the cause is decisive for the place of origin of the constrictions. As already said, we *must* (italics mine) conceive of this cause as 'Fernkräfte,' more particularly as 'Fernkräfte' acting at a particular distance. But the distance of the effect (which is inherent) is not absolute, because in gastrulae of any size, produced by operations, the intestine segments equally well into the proper proportions; thus the effective distance of the 'Fernkräfte' acting from one end point of the system is given not as an absolute but as a relative amount, being dependent on the length of the axis of the differentiating system.

"The establishment of heterogeneity affords new places for new kinds of 'Fernkräfte,'" *und so weiter.* Driesch passes here from saying in one paragraph we *can* represent the process by *assuming* "Fernkräfte" acting from a fixed point, to the assertion in the next that we *must* conceive of the cause as "Fernkräfte." Hereafter, then, he regards his postulate as a logical necessity, no longer a mere intellectual convenience, like Maxwell's demon, although it is an hypothesis of a precisely similar nature.

It is pertinent to ask whether in any extremity the assumption of "Fernkräfte" is permissible; but, passing this by, the postulated force must either be a product of the system or something independent of the system. If a product of the system, when all parts are alike, it must be postulated of each part; each element then is not only bilaterally symmetrical, but has a "Fernkraft" "seated" at one of its poles; as soon as the heterogeneity demanded by this is established, the elements in each section become endowed with new kinds of "Fernkräfte." Unless these arise absolutely *de novo* (!), they must have existed in a latent form previously, and there would appear to be no escape from the conclusion that each element of

the protoplasm must be endowed originally with all the "Fernkräfte" of every localization of the entire ontogeny. But are the "Fernkräfte" independent of the system? What sort of science would this be? Yet Driesch appears, without actually discussing these alternatives, at times to lean to this view. For instance, p. 95: "The actual adaptiveness of the process *appears* thus in this case to be contained *both* in the causing spot (verursachenden Ort) and the forces proceeding from it, *as well as* in the system concerned." He explains this "appears," on the next page, by saying that in reality the conception of adaptiveness belongs only to the system, because this controls by its absolute size the effective radius of the "Fernkräfte."

Does the conception of "Fernkräfte" reduce itself to absurdity or does it not? One must admit that it is often difficult to extract the meaning from the welter of words, and so I may have misinterpreted Driesch, though I have honestly tried to understand and represent his ideas fairly. Driesch believes his theory of "Fernkräfte" to be rational vitalism; I should prefer to style it irrational mysticism.

On the other hand, the theory of the bilaterality of the protoplasmic elements appears to me a useful hypothesis, even though it may be difficult to see that their endowment with bilaterality alone is a logical halting ground. In the case of the egg of *Unio*, at least, the theory would seem to be of greater use if we were also to postulate of the elements certain antero-posterior proportions.

That the entire organism in every stage of its development exercises a formative influence on all its parts appears to me an absolutely necessary hypothesis. Only thus can we explain the reorganization of portions of the egg or of the adult into complete individuals, or form a satisfactory picture of the maintenance of individuality. The theory of division of labor needs reinforcing by a companion principle of unity of organization. The comparison of the metazoan body to a community or state is inadequate; it is this inadequate conception that has led to the extreme cell theory of development, as upheld by Hertwig and the majority of zoölogists. The misconception may be traced back to Haeckel's comparison of the

egg to a protozoan, and the cleavage stages to colonies of protozoa. We are even beginning to doubt that *phylogenetically* the protozoan-colony theory is a necessary view of the origin of the metazoan body; ontogenetically this view has become impossible.

But though the hypothesis I have just stated above appears necessary to many at the present time, I think there are very few who will follow Driesch in his rapid flight from one extreme, the dominant cell theory which he upholds in his earlier papers, to the opposite: that only incomprehensible and intangible vital forces, such as "Fernkräfte," are adequate to explain the unity which, as we are beginning to see, pervades all the metamorphoses of any species. I think that most naturalists will resolutely suppress the feeling, almost of panic, that accompanies the revelation of the real complexity of the problems of development, and will stand firm in the unassailable position that, until it is shown that there enter into the composition of protoplasm elementary substances not known elsewhere, vital properties must be explained as in some way due to the qualities of that matter which physicists and chemists investigate. On the other hand, better understanding of the marvelous properties of protoplasm furnish problems to the physicist and must inevitably lead to a reconsideration of the properties and theories of matter.

Zur Strassen ('98) has been led by his studies on nematode development to reject mechanical views of cleavage. He is impressed by the adaptive nature of the earliest cleavages and exclaims: "It is as though the cleavage cells possessed a perfect guiding *instinct*." "Ich zögere wirklich kaum, diesen Begriff allen Ernstes für das Verhalten der Blastomeren in Anspruch zu nehmen." He gives a vivid description of the manner in which the *T*-shaped early 4-cell stage is converted into a rhomboid by active amoeboid migrations of the cells, sometimes carried out in the face of considerable difficulties, as in the giant eggs, where the cells have to force past a constriction of the egg-membrane. "Ich sah zwei Organismen ein Ziel, ihre Vereinigung, erstreben und dieses Ziel erreichen. . . . Die Zellen sind nicht Bausteine der Entwickelung, die durch

fremde Kräfte geformt und an ihren Ort geschleppt worden, sondern Steine und Baumeister zugleich."

The use of the term "instinct" for such phenomena of irritability and response as the parts of an embryo exhibit is questionable, and is not in any case very illuminating. While I heartily agree with Zur Strassen that we must look within the egg for the chief causes of embryo formation, it appears to me that he attributes too much to "social instinct" of the cells. In this he resembles Hertwig, indeed, sometimes using similar expressions, as where he speaks of single cells of the 4-cell stage as "organisms." The four cells form but one organism; "it is neither functional *economy* nor social instinct that binds the two halves of the egg together, but the constitutional bond of *individual organization*" (Whitman, '93, p. 115).

The view that I have attempted to demonstrate is: 1. That the cytoplasm of the egg of *Unio* possesses a definite organization involving bilateral symmetry and certain antero-posterior proportions; 2. That this is not primarily dependent on localization of specific substances, but in some way results from the interaction of the idiosomes.

I believe: 1. That this particular kind of organization is continued throughout the entire life cycle, and is one of the main factors in axial regeneration where this occurs; 2. That in every stage of the organism it is an undivided force. It therefore implies protoplasmic continuity throughout.

I regard this as one of the most important factors in ontogeny, and think it important to clearly distinguish it from the other factors, such as reactions of nucleus and cytoplasm, tropisms and taxic phenomena, and all relations of parts. It differs from these in being the property of the cytoplasm as a whole.

It appears to me that the problem we are considering recurs in fundamentally the same form in the reorganization of a fragment of a stentor or a planarian into a complete individual. The materials in which the formative forces[1] are at work are different in these cases, and so there are different modes through which they reach their expression. But whether these forces are working through adaptive cleavage, or in a

[1] I use this term in a purely descriptive sense.

mass of undifferentiated cells, such as the chick blastoderm in an early stage, or in a mass of material composed of specific tissues, as in a portion of a planarian, it appears to me that they are in principle everywhere the same; I would say that the same cytoplasmic organization is concerned alike in the development of the planarian from the egg and from the fragment of a mature individual. Where are we to place it except in those protoplasmic elements of whose nature we are so ignorant?

"If the formative processes cannot be referred to cell division, to what can they be referred? To cellular interaction? That would only be offering a misleading name for what we cannot explain; and such an answer is not simply worthless, but positively mischievous, if it puts us on the wrong track. Loeb's experiments on heterogenesis furnish a refutation of the interaction theory. The answer to our question may be difficult to find, but we may be quite certain that when found it will recognize the regenerative and formative power as one and the same thing throughout the organic world. It will find, as Wiesner has so well insisted, a common basis for every grade of organization, and it will abolish those fictitious distinctions we are accustomed to make between the formative processes of the unicellular and multicellular organisms. It will find the secret of organization, growth, development, not in cell formation, but in those ultimate elements of living matter, for which *idiosomes* seems to me an appropriate name." (Whitman, "The Inadequacy of the Cell Theory of Development.")

I think that we need not ascribe to the egg a more complex organization than it may be ultimately possible to discover in it by observation and experiment, and should attempt to explain the subsequent development, phenomenally at least, in a purely epigenetic manner. I am very far from asserting that in the case of the egg of *Unio* we have fully described the organization in the cytoplasm, to say nothing of the nuclear material. But what I do mean to assert is that we should rid ourselves of all mysticism in dealing in a purely scientific manner with the problem of the organization of the egg, or the nature of the

primary formative forces. These should be matters of pure description of facts observed, or inferences of a descriptive nature directly drawn from these facts; theories of *potency* should be left to the metaphysicians. No amount of observation or experiment will enable us to add anything to metaphysical theories of potency; if we are to devote ourselves to these, we might as well shut up our laboratories.

It does not appear to me that in the above sense there is any insoluble problem in the whole field of heredity. We may hope, by sufficiently long, patient, and searching investigation, some day to be able to trace the organism through all phases of its metamorphoses from the egg of one generation to that of the next, to describe the exact nature of the organization that is continued throughout, to estimate at its proper value the influence of external conditions at each step, and so solve the old riddle of evolution and epigenesis, and that of the influence of the soma on the germ. That there is continuity of organization we know, but what the organization that is continued may be, we do not know. This is the question that presses most at the present time for solution.

The solution of the problem of differentiation would be given by an *exact* description of *every* step in the process. If this ideal should ever be realized in any one case, there would be no further need of *scientific theories* of development. But it seems extremely improbable that it can ever be actually realized, though I believe that it will be possible by sufficiently detailed observation so closely to approximate what we call stages of development to each other that but a single rational theory will exist capable of filling up the gaps. Therefore, observations such as those of Boveri ('99), Conklin ('99), and those described here seem to me the surest means of approaching this theory. That experimental methods will be of immense service no one can doubt. The study of living material will also be an indispensable method.

In conclusion, then, I would state as my opinion that the organization that is continued from one generation to another consists of nuclear material in a mass of cytoplasm possessing a definite orientation, the extent and nature of which must be

left to future investigation; I have described here only what seemed to me necessarily to follow from the phenomena accompanying the maturation, fertilization, and cleavage in *Unio*. It is this orientation in the cytoplasm that gives a differential value to mere position (Driesch), for "position" in an unorganized mass of cytoplasm is unthinkable. We have sufficiently considered the evidence for believing that this organization must be a property of the idiosomes; and it seems to me at present that Driesch's conception of bilaterality of these elements is likely to prove a fruitful one.

VASSAR COLLEGE,
May, 1900.

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DESCRIPTION OF PLATES.

All figures are from camera drawings of sections of the eggs of *Unio complanata*. Most of the figures are from single sections, but when two or more sections are combined in a single drawing this is always stated in the description. The lenses used for the drawings were Zeiss Apochromat 2 mm. Homog. Immers. and compensating ocular No. 6, except where otherwise stated. The figures are all drawn from eggs killed in Boveri's picro-acetic mixture, and stained on the slide with Heidenhain's iron-haematoxylin, followed or preceded by Bordeaux red in aqueous solution. Needless to say other methods were used, but the drawings are not published. The sections were 5 or 6 2/3 μ in thickness.

EXPLANATION OF PLATE XXIV.

FIG. 1. Radial section through the egg. Early stage of the first maturation spindle. Sperm-head and comet-like sperm-amphiaster.

FIG. 2. Perfect axial section. Part of the egg-membrane with the micropyle is shown. Somewhat later stage of the first maturation spindle than in Fig. 1. The entire path of the spermatozoön is shown. Peripheral distribution of yolk-granules. The section was injured before the completion of the drawing, so the maturation spindle is represented somewhat diagrammatically.

FIG. 3. Sperm-nucleus in the same position as in Figs. 1 and 2; but the sperm-centrosome is just dividing.

FIG. 4. Sperm-head and amphiaster from a stage of maturation similar to Fig. 2. The plane of separation of the sperm-centrosomes is nearly at right angles to the penetration path. Many similar eggs found. The distal sperm-centrosome is divided in three parts.

FIG. 5. Turning of the first maturation spindle; stage intermediate between Figs. 1 and 2. Combination of two successive sections.

FIG. 6. Section of unfertilized egg with first maturation spindle. In the other eggs of the same lot, which were fertilized, the first polar globule was already fully formed, and the second in process of formation.

FIGS. 7 *a* and 7 *b*. Sperm-nucleus and sperm-aster from different sections of the same egg, in a stage of maturation intermediate between Figs. 2 and 5. The centrosome in 7 *a* is attached by a fiber to the tip of the sperm-nucleus, and the aster is gone. The aster around the centrosome in 7 *b* is already breaking up.

FIG. 8. Slightly later stage of degeneration of the sperm-asters. But one centrosome could be found; the one that originally lay nearer to the sperm-nucleus is entirely indistinguishable.

FIG. 9. Radial section of the first maturation spindle at the time of the metaphase; one of the chromosomes has already divided transversely. Part of the inner aster was drawn from another egg, where it showed more plainly.

FIG. 9 *a*. Sperm-head in the same egg from which Fig. 9 was drawn. The sperm-centrosomes are now no longer distinguishable; and the clear area has

become much smaller. The sperm-nucleus is contracting, and may be seen to be composed of about sixteen chromatic vesicles. Only those of one surface shown.

FIG. 9*b*. Sperm-nucleus of another egg in the same stage of maturation, to show the vesicular structure.

FIG. 10. Tangential section through the outer aster in the stage of Fig. 9, to show the concentric spheres, inner and outer, and the rays penetrating to the dumb-bell-shaped centrosome.

FIG. 11. First maturation spindle just before the beginning of the protrusion of the first polar globule. The centrosomes at each end are double, and the chromosomes are assuming the form characteristic of the second maturation division, thus repeating their form in the first maturation spindle. The microsomes are beginning to grow large in the interzonal region where the stress has ceased. *Cf.* Fig. 8.

FIG. 12. First maturation spindle. In this stage the two centrosomes of each inner sphere are distinctly compound, double or quadruple. The enormous size of the microsomes in this stage is especially noticeable. The chromosomes have completely assumed the form characteristic of the second maturation division.

FIG. 13. Constriction of first polar globule. The inner sphere and group of chromosomes have moved bodily near to the surface, to which they are attached by a new set of radiations. The sphere remaining in the egg is smaller than in the preceding stage (Fig. 12), and the centrosomes within it are closer together.

FIG. 14. The first polar globule is fully formed, but is still attached by means of "Zwischenkörper" fibers to the surface of the egg. (These were found in the next section, which is not figured.) The centrosomes of the egg-sphere are now in contact, and the radiations which in Fig. 9 extend from the sphere to the surface have now been resolved into vesicular cytoplasm.

EXPLANATION OF PLATE XXV.

FIG. 15. Earliest stage in the formation of the second maturation spindle. The central spindle arises within the inner sphere. Each of the centrosomes is at least quadruple. Radiations surround the entire sphere, and are not specially related to the centrosomes of the new central spindle. The fibers of the aster of the first maturation spindle form a reticulum with large, deeply staining microsomes.

FIG. 16. Slightly later stage; the elongation of the central spindle has stretched the sphere into an elliptical form. The boundary of the sphere forms the periphery of the new central spindle, from all parts of which radiations proceed.

FIG. 17. Further elongation of the central spindle; the radiations of the preceding stages (Figs. 15 and 16) have become resolved into vesicular cytoplasm, and new asters are forming in this from each of the compound centrosomes; the aster at the inner end of the spindle is much farther developed than that at the outer end. Mantle-fibers are attached to the chromosomes, which are being drawn towards the equator of the spindle. The centrosomes are larger and more subdivided than in the preceding stages, but as they are somewhat flattened radially this is not very evident in this figure. See, however, Fig. 24.

FIG. 18. Later stage. The chromosomes are now nearly at the equator of the spindle. The inner centrosome has nearly undergone the metamorphosis more fully pictured in Figs. 24–27. The outer end of the spindle is in contact with the surface, apparently as a result of the elongation of the spindle; fibers pass from the inner centrosome to the surface.

FIG. 19. In this stage the outer centrosome still consists of a number of deeply staining granules, each of which under ocular 18 seems to be compound. The outer sphere is now established by the arrangement of the first row of microsomes.

FIG. 20. The outer centrosome has now completed its metamorphosis into a sphere (inner sphere) with central granule (centrosome). The chromosomes are splitting longitudinally. For the sake of clearness only about one-third of the chromosomes are drawn in.

FIGS. 21–23. Metaphase and anaphase of the second maturation spindle. Note the peripheral distribution of the yolk in Fig. 23.

FIGS. 24–27. These are tangential sections through the outer centrosome of the second maturation spindle in the stages of the spindles figured immediately above them (Figs. 17–20). They are camera drawings with comp. oc. No. 8 and hom. imm. apo. 2 mm. of Zeiss.

FIG. 28. *Accessory amphiaster.* The inner end of the second maturation spindle (in the anaphase), cut obliquely, is to the right. The sperm-nucleus in this egg was found in another section near the side of the egg opposite to the accessory amphiaster.

FIG. 29. Figs. 29–31 illustrate the second mode of formation of the second maturation spindle. In Fig. 29 the aster and centrosome of the first maturation spindle have almost entirely disappeared.

FIG. 30. The chromosomes have moved in towards the center of the egg, and the central spindle is forming in a horizontal plane. Each end of the spindle is occupied by a group of centrosome granules.

FIG. 31. The spindle is completely formed and has swung into its definitive radial position. At each end of the spindle is a large group of centrosome granules, which later undergo the metamorphosis shown in Figs. 24–27. There is no such difference in the time of the metamorphosis of the centrosomes in this mode of formation of the spindle as in the other mode.

EXPLANATION OF PLATE XXVI.

FIG. 32. Telophase of the second maturation spindle. The inner sphere has enlarged considerably, and in place of the single centrosome there are a number of deeply staining bodies united by a delicate reticulum within the sphere. The entire sphere stains more darkly than before. The aster shows the usual appearance of degeneration, being thickly studded with large microsomes.

FIG. 33. Beginning of reconstruction of the egg-nucleus. The inner sphere has swollen up to a relatively enormous size, and is occupied by a loose-meshed reticulum with deeply staining microsomes on the fibers.

FIG. 34. The egg-nucleus has enlarged considerably, and the sphere substance is continuous with the general cytoplasm.

FIG. 35. Beginning of the migration of the germ-nuclei. The egg-nucleus, accompanied by the sphere substance, is moving towards the side of the egg away from the sperm-nucleus, which has taken up its march in the general direction of the egg-nucleus. Zeiss 4/2 mm. Combination of four sections.

FIG. 36. Similar section in a slightly later stage of the migration. In this stage the sphere substance has elongated in a plane at right angles to a line uniting the germ-nuclei and to the axis of the egg. Zeiss 4/2 mm.

FIG. 37. Section in the plane indicated on Fig. 36 to show the elongation of the sphere substance.

FIG. 37*a*. Outline of sphere substance in a later stage stretching completely across the egg.

FIG. 38. Meeting of the germ-nuclei. This is the earliest stage in which the cleavage centrosomes can be distinguished. The figure is a combination of two successive sections; hence the appearance of overlapping of the nuclei. *The centrosomes are therefore simply seen through the nuclei. They do not lie in them.* For the sake of clearness the chromatin has been omitted, except in the right-hand border of the egg-nucleus. The latter can be distinguished from the sperm-nucleus by its relation to the sphere substance, which can still be distinguished.

FIGS. 39–43. Illustrate origin of asters, spheres, and chromosomes of the first cleavage spindle. The position of every chromatin granule and spindle fiber in Figs. 39–43 is drawn in as accurately as possible.

FIG. 39. Combination of two successive sections; before the formation of the central spindle. The centrosomes are broken into a number of parts imbedded in a red-staining mass. Outer sphere well marked; mag. Zeiss 8/2 mm.

FIG. 40. Slight advance in aster and spindle formation. First rudiments of chromosomes may be distinguished; mag. Zeiss 8/2 mm.

FIG. 41. Origin of central spindle; chromosomes and mantle-fibers forming. Notice chromatin masses near the centrosomes. Slight diminution in amount of basichromatin. Zeiss 8/2 mm.

FIG. 42. Combination of two successive sections. Great diminution in amount of basichromatin; long chromosomes appearing. Other chromatin granules in spindle fibers, and yet others in the neighborhood of the centers. Remainder in the form of oxychromatin (?). Zeiss 8/2 mm.

FIG. 43. Early stage of the first cleavage spindle; the two groups of chromosomes readily distinguished; each chromosome is very long and delicate but distinctly double.

FIG. 44. Later stage of the first cleavage spindle before its shifting. Vesicular "centrosome" (inner sphere).

EXPLANATION OF PLATE XXVII.

FIG. 45. Shifting of first cleavage spindle to one end of the egg. Observe that the spindle does not elongate as it moves. Stage immediately preceding the metaphase.

FIG. 46. Metaphase of the first cleavage spindle; for the sake of clearness only about one-third of the chromosomes in the section were drawn. In this stage there is a single centrosome within a hollow inner sphere. The egg is perfectly round.

FIG. 47. Anaphase. Egg slightly elongated in the plane of the spindle. The inner sphere is enlarged and occupied by a delicate reticulum.

FIG. 48. Beginning of telophase; the spindle has shifted to its definitive position; the line of shifting is plainly marked. The outer sphere has disappeared; the inner spheres have enlarged yet further, and are occupied by a reticulum with microsomes at the nodes.

FIG. 49. Combination of two sections. Constriction in first cleavage plane begun. The inner sphere has grown much more and altered its form; its reticulum is wider-meshed. The remains of the aster is a close-meshed reticulum (or foam). The migration of the sphere substance in the larger cell is already indicated.

FIG. 50. Combination of two sections. The sphere substance has lost its sharp boundaries, and in the larger cell is migrating towards one side of the cell which is elongating in that direction.

FIGS. 51 and 52. Two sections of the larger cell parallel to the first cleavage plane in a slightly later stage. The nucleus is yet in process of reconstruction, and the sphere substance is stretching across the cell. The furrow is on the vegetative pole surface of the cell.

NOTES ON REGENERATION AND REGULATION IN PLANARIANS[1] (*continued*).

By FRANK R. LILLIE.

THE experiments herewith recorded have been performed as occasion offered and have consequently extended over a considerable period of time. They have been selected from a large body of notes, because the facts are themselves new, and, the experiments being made on genera not hitherto studied from this point of view, the results may serve to control the theoretical deductions made from the study of *Planaria*, the genus of aquatic triclad Turbellaria hitherto exclusively studied.

II. Regeneration of the Head in Dendrocœlum Lacteum.

In Dendrocœlum, which is closely related to Planaria and similar in structure, the capacity for regeneration is very much less, and as concerns the head is limited to the anterior third of the body, approximately, though a new tail may regenerate at any transverse level.

My first experiment was to cut a single specimen in two through the pharynx, July 23, 1899. The cut surface healed, and the farther fate of the parts was as follows. The posterior part formed no new tissue, although it lived for twelve days. From the anterior portion, on the other hand, there grew out a pointed piece, which formed a tail. I afterwards repeated the experiment several times with a similar result. Thinking that the failure to regenerate a head might be due to the presence of the pharyngeal pouch, I then cut fourteen specimens transversely immediately behind the pouch. Two days after the operation I noticed an interesting difference in the reactions of the two kinds of parts. While the anterior pieces reacted in all respects like intact individuals, migrating to shaded parts of the dish, the posterior parts showed no definite reaction of this sort, but re-

[1] Number 1 of these notes, "The Source of Material of New Parts and Limits of Size," appeared in volume xxxiv of the American Naturalist, March, 1900, pp. 173–177.

mained scattered irregularly over the bottom, some with the ventral surface up. In four days five of these pieces died, in five days two more were dead, and in six days all of the posterior pieces were dead without having shown any signs of regeneration; while all of the anterior pieces, kept in the same dish, were living and regenerating new tails. This experiment was repeated with similar result, and I soon became convinced that, in this form, while a tail might regenerate at any transverse level from the pharynx back, a new head could not be formed from tissue behind the pharynx.

The question now was, could a new head be formed in front of the pharynx at any level? Specimens were cut transversely immediately in front of the pharynx. The result of these experiments was that, while a very narrow border of new tissue might be formed at the cut end of the posterior pieces, there was never regeneration of even the semblance of a head.

In the next experiment the heads of five individuals were removed by a transverse cut just behind the eyes. The heads, thus removed, did not regenerate, but in five days it was apparent that new heads were forming on the decapitated pieces, and in one of them the eyes could already be seen. In six days new eyes could be seen in all. The capacity for regeneration of a head was thus demonstrated.

Two questions now remained: first, is the development of a new head due to the position of the cut, or to the size of the piece? and second, how far back does the capacity for regeneration of a head extend? The first question received an answer in a very simple way. The head was first cut off just back of the eyes, and then from the anterior end of the major piece a small transverse part was cut. In six days a rudimentary head with eyes developed on one of three such small transverse parts. (The two parts that did not form heads were probably cut too small.)

How far back does the capacity for regeneration of a head extend? We have seen that it cannot be formed from tissue just in front of the pharynx, but that it can be formed just back of the eyes. Twelve specimens were cut transversely about half-way between the anterior end of the pharynx and the tip of the head. The reactions of both kinds of parts were quite normal, though the headless parts reacted much less rapidly than the head-bearing ones. In seven days both parts were rapidly regenerating, and eyes had appeared in the posterior parts; in each head-bearing part a new pharynx and tail were forming. Both kinds of parts then completed

the regeneration rapidly, though in nineteen days the normal proportions were far from being restored.[1]

Thus in Dendrocœlum, while tissue may grow out at any transverse level, behind the region immediately back of the eyes, in the form of a tail, the capacity for regeneration of a head is limited to the anterior third or fourth of the body. I do not mean, of course, to state that the formation of a head back of this region is completely impossible. Some one may, at any time, demonstrate by operating on a sufficiently large number of individuals, that a head may exceptionally regenerate back of this level. But these experiments demonstrate very clearly that the power to regenerate a new head is limited in Dendrocœlum, by other conditions than size of the piece or presence of certain parts of the intestine.

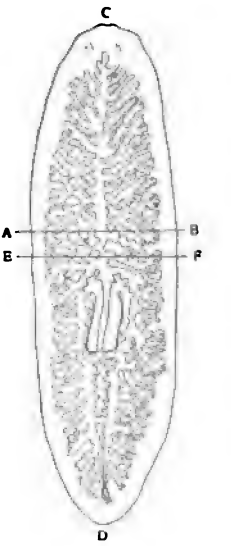

FIGURE 1. — Exact camera drawing of a stained specimen of Dendrocœlum. Note extensive anastomosis of gut-diverticula especially on left side. In other specimens more than one transverse anastomosis between posterior gut rami may occur.

Dendrocœlum differs in this respect from both Planaria and Phagocata, but strangely enough resembles the earthworm *Allolobophora fœtida*. In this form, according to Morgan's[2] observations, regeneration of a head does not ordinarily occur back of the fifteenth segment and never behind the middle of the body, although a new tail may regenerate at any level back of the tenth segment.

Why is it that embryonic tissue will continue to grow and differentiate on the posterior end of ABC (Fig. 1), but not on the anterior end of ABD? It is not because the cells of the ectoderm or of the mesenchyme or of the gut are incapable of growth

[1] This point deserves emphasis. In Planaria and also in Phagocata regenerating parts rapidly assume the normal proportions, after the differentiation of the new tissue; this involves great changes in the form and positions of the organs. The completely regenerated individual thus comes to possess not only all the parts of a normal worm, but also the same proportions. Morgan [Biological Lectures from the Marine Biological Laboratory, Woods Holl, 1899, Boston: Ginn & Co., 1900] has proposed the term "morphallaxis," for this phenomenon of translocation of tissues by which the normal proportions are reassumed. In Dendrocœlum, morphallaxis is very slight, or in some parts entirely lacking.

[2] MORGAN, T. H.: Archiv für Entwicklungsmechanik der Organismen, 1897, v, pp. 570–586.

and differentiation at the level AB, because the same tissues, ABEF, that grow out into a tail, if forming part of CEF, will not grow at all if forming part of ABD.

The explanation of this peculiar fact must lie in some conditions of the piece ABC not found in ABD. I believe that the determining condition is the presence of the brain and anterior part of the central nervous system in the anterior piece.

How may the presence of this part of the central nervous system be thought to influence regeneration? The possibilities would seem to be:—1. That it may exercise a "trophic effect." 2. That it may act by coördinating the activities of the piece, and in consequence establishing normal stimulation in the regenerating part. 3. For regeneration of a brain we might suppose that certain cells not found in sufficient number back of a given region are necessary.

All of these possibilities presuppose differences, which actually occur, between the central system of Dendrocœlum on the one hand, and of Planaria and Phagocata on the other, cephalization being more advanced in Dendrocœlum than in the other genera.

I am unable to determine whether the brain does or does not exercise a "trophic effect," or whether the statement that it does would have any different meaning from what follows. Certain facts concerning the reaction of parts of Dendrocœlum incapable of regenerating a head lead me to place particular emphasis on the second possibility. Some years ago Loeb made the interesting observation, often since repeated (see Parker),[1] that decapitated specimens of Planaria react to the usual stimulus of light like normal individuals, but more slowly. Any symmetrical piece of Dendrocœlum capable of regeneration tends to come to rest in the shaded parts of the dish precisely like a normal individual. No doubt the coördination of movements upon which so definite reactions depend, is regulated by the portion of the nervous system within the piece. However in Dendrocœlum, all parts incapable of regenerating a head also become incapable, after a day or two, of performing the usual reaction to light. Thus my notes concerning twelve specimens of Dendrocœlum cut in two immediately behind the pharynx read: "Two days after the operation all of the anterior pieces go to the side of the dish and remain in the angle between the bottom and side; but none of the posterior pieces

[1] G. H. Parker and T. L. Burnett: This journal, 1900, iv, p. 373.

shows such reaction; these remain scattered over the bottom, about half of them with the ventral surface up." I have other similar records in my notes; but also some in which the reaction seems to have continued normal.

However, there seems to me to be a connection between the lack of correlation of movement and the inability to regenerate a head.

It is therefore quite possible that the fate of the undifferentiated mass may be determined by the stimulation of the normal movements; if at the anterior end, the forward extensions and contacts may furnish such stimuli; if at the posterior end the stimuli would be of a different nature. In fact, the stimuli would differ for all variants in position of the differentiating mass.

But, though I am led by the conditions found in Dendrocœlum to attach particular importance to these normal stimuli, I certainly agree with Bardeen[1] that the internal conditions prior to regeneration are of great importance. It seems to me that earlier observers have erred chiefly in not paying attention to the conditions of the internal parts.. It is especially the physiological actions of these parts that must be taken into account. Bardeen has insisted on this in the case of the intestine principally. But similar relations obtain also in the muscular system and in the nervous system. The contraction of the muscular coats of the body wall is of the nature of a peristaltic wave. In any piece therefore different conditions, brought about by the direction of the contraction-waves, would obtain at anterior and posterior ends. The direction of the nervous impulses within the portion of the nervous system contained in the regenerating part would be typical. Thus in the gut, in the musculature, and in the nervous system the anatomical and physiological relations clearly distinguish anterior and posterior ends. Possibly similar anatomical and physiological conditions prevail in other parts. Before we have recourse to such dimly conceived hypotheses as "polarity," the possible differential effect of known anatomical and physiological conditions should be considered.

It seems to me, therefore, that the factors determining the fate of embryonic tissue at the anterior and posterior ends respectively are probably of this nature: — in the first place, the actual anatomical and physiological relations of parts, in all of which in any transverse part there is some antero-posterior differentiation; in the second

[1] BARDEEN, C. R.: This journal, 1901, v, p. 1.

place, the coördination of all organs comprising a separated piece, depending probably on the nervous system, and leading to normal stimulation of any exposed embryonic tissue.

III. Regeneration of the Pharynges of Phagocata.

The specimens of *Phagocata gracilis* with which I experimented were found in the same pond with *Planaria maculata* and *Dendrocœlum*. The manner of life is very similar to that of the other two genera. Apparently it does not reproduce by fission. The power of regeneration of this genus is equal to that of Planaria; for instance, a single specimen may be cut into sixteen parts capable of complete regeneration. Phagocata, as is well known, possesses a very long pharyngeal pouch (Fig. 2), containing a large number of pharynges; one of these occupies the usual position at the anterior end of the pouch, while the others are attached laterally and communicate with short branches of the posterior gut rami.

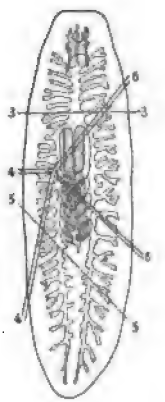

FIGURE 2. — Phagocata gracilis (slightly diagrammatic). The lines 3–3, 4–4, 5–5, 6–6, indicate approximately the source of the parts shown regenerated in Figs. 3, 4, 5, and 6.

a. In most parts, after three or four days, several pharynges are found regenerating simultaneously, though the more anterior ones are more advanced, and posteriorly the earliest rudiments are found (Fig. 3).

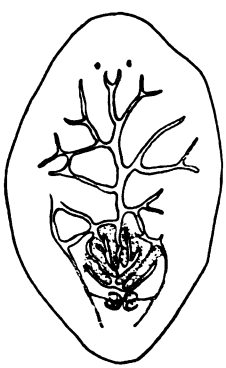

FIGURE 3. — Phagocata gracilis. Regeneration of the part in front of the line 3–3 (Fig. 2).

The formation of the pharynx and pharyngeal pouch from the mesenchyme has already been noticed by Woodworth[1] for Phagocata and Bardeen for Planaria. The former author gives a detailed account. The first rudiment is an accumulation of cells of embryonic type; near the periphery of this a semi-lunar cavity appears and forms the rudiment of the pouch; the tissue projecting into the pouch differentiates into the pharynx. The mouth is a secondary perforation of the floor of the pouch. It may be worth

[1] Woodworth: Bulletin of the Museum of Comparative Zoölogy. Harvard, 1891, xxi, p. 1.

while to notice in passing that this differs from the ontogenetic method of origin, both pharynx and pouch arising in the latter case from an ectodermal invagination.

b. The origin of the pharyngeal pouch as a cavity in the mesenchyma surrounding the free end of the pharyngeal rudiment has the following curious effect: the definitive common pouch arises as a series of separated cavities, which secondarily fuse together. The original partitions are recognizable for a long time as pointed projections of tissue into the pouch between the pharynges, Figs. 3 and 4. But in some cases of regeneration the fusion is incomplete or entirely absent, so that two or more pouches may be present, each containing several pharynges, Fig. 4. A curious abnormality is represented in Fig. 5: two pharynges have a common termination; evidently two buds arose very near together and partly fused.

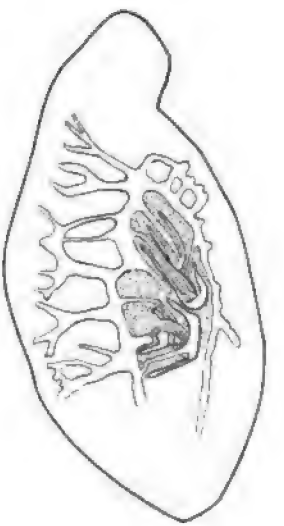

FIGURE 4. — Phagocata gracilis. Regeneration of the part bounded by the lines 4–4 (Fig. 2).

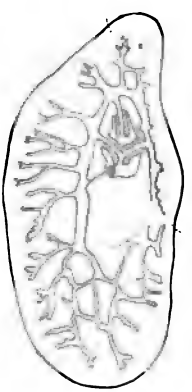

FIGURE 5. — Phagocata gracilis. Regeneration of the part behind the line 5–5 (Fig. 2).

c. As indicating that there is a special relation between the regeneration of the gut and the pharynges, the regeneration of lateral pieces offers some features of interest. The pharynges form near to the cut edge, as does the single pharynx in Planaria. Fig. 4 shows a case of this sort; there is a long pouch near the cut edge, in this case divided in two parts by a partition, and all of the pharynges are on the side towards the old tissue, with a single exception. Connecting with each pharynx, however rudimentary, is a branch of the intestinal system. The pharynges never begin to develop in such pieces on the outer side of the pouch until a branch from the intestine supplies the neighborhood. It will be seen that a new branch, destined to form the posterior ramus of the regenerating side, has grown along outside the pouch. This branch is very slender, but in connection with it a new pharynx has already differ-

entiated; other pharynges form along this side later. But in many observations made on the regeneration of lateral pieces, I never found the regeneration of pharynges along the cut side begun, until the posterior ramus of the intestine of the same side was formed. (See also Fig. 6.)

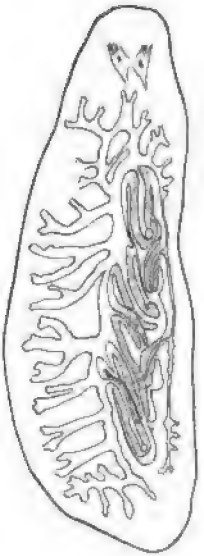

FIGURE 6. — Phagocata gracilis. Regeneration of the part bounded by the lines 6–6 (Fig. 2).

d. Usually the regeneration of the various parts takes place in a coördinated fashion, the pharynges developing in relation to the intestine, etc., and all parts in such a manner that normal, *i. e.*, adaptive, relations are established. Fig. 7 shows an exception to this rule. The part figured was as nearly as possible a $\frac{1}{32}$ part of a normal individual; the individual in question was divided into sixteen equal transverse pieces, and each of these was cut through its centre. None of these parts regenerated completely. Their history was as follows. Operation, Aug. 1st, 1899, thirty-two pieces; Aug. 2nd, twenty-one pieces living, the smaller pieces very thick dorso-ventrally; Aug. 4th, two pieces living. These were killed and stained, and a drawing of one of these is shown in the figure.

FIGURE 7. — Phagocata gracilis. Regeneration of a $\frac{1}{32}$. The gut branches were very broad, irregular, and branching in more than one plane; the superimposed branches are not represented.

The points of interest are that, while the piece has not regenerated a whole, the intestine has grown out in all directions through the piece, and at each of two places a pharynx has regenerated. These pharynges are widely separated and turned in opposite directions, a species of heteromorphosis. The side toward which they lay was evidently the injured side. Certain regenerative processes may go on in such a piece, but there is not full coördination, and the result is non-adaptive.

IV. Theoretical and Critical.

The phenomena of regeneration offer many problems, some of which not only appear insoluble in the present state of our knowledge, but actually offer no point of attack. For instance, if one were to ask why the regenerating rudiment of the head develops into such different forms in Planaria, Dendrocœlum and Phagocata, it could only be said that we do not know; nor are we able to say why Phagocata has many pharynges and the other genera of planarians only one. No physiological explanation of these phenomena can at present be offered. But if we leave out of account such problems, there still remain certain problems of regeneration that may be attacked with good expectation of success. The localization of regenerating organs is one of these. In the case of the regeneration of Hydroids, Loeb[1] has treated this question with great success, by showing that the regeneration of hydranths or rhizoids depends on certain external stimuli, such as, in different cases, light, gravitation, or contact.

A recent paper by Bardeen[2] seems to me to make for the planarians a decided advance in this direction inasmuch as he pays particular attention to the relations of the regenerating parts to the organs originally present in the piece. I propose, therefore, to devote some attention to Bardeen's theory in the light of experiments described in the second and third of these notes, which deal with different genera of planarians from the one (Planaria) used by Bardeen. I shall therefore first state his conclusions at some length.

1. "Embryonic tissue is formed in the specimens here studied at two places only, (1) at or near a cut surface, and (2) in the region of the piece just posterior to the point of least intestinal pressure." The author offers the following suggestions as to why the embryonic tissue should be formed at these places. The formation of embryonic tissue at the cut surfaces may be due to one or other of these causes: (1) slight change in the osmotic pressure; (2) exposure of the internal tissues to the water; (3) enzymes set free by the injury.[3] "The cause of the formation of embryonic tissue just pos-

[1] Loeb, Jacques: Biological Lectures Delivered at the Marine Biological Laboratory of Woods Holl, 1893, p. 37, Ginn & Co., Boston, 1894.

[2] *Loc. cit.*

[3] Loeb: This journal, 1900, iv, p. 60.

terior to the point of least intestinal pressure is equally dark. The process is much slower and is preceded by a retrograde metamorphosis in the pre-existing adult tissue. We might assume that here certain intestinal fluids are set free by pressure."

2. "The differentiation of this embryonic tissue depends on its relations to the intestinal apparatus of the animal. (1) If it lies anterior to the main axial gut, it becomes converted symmetrically into a head region." (2) "If the embryonic tissue lies lateral to the axial gut or to a line extending directly posterior to this, it becomes converted into a new lateral region." (3) "If the embryonic tissue lies at the posterior end of the axial gut, *i. e.*, behind the point of least intestinal pressure, it becomes converted into a new pharynx and pharyngeal pocket." (4) "If the embryonic tissue lies posterior to the pharyngeal region, it becomes converted into a new tail."

The author concludes from these facts that the intestinal system has a specific action in determining the nature of the parts developed from the embryonic tissue, rejecting the idea that the development of the intestinal system may be merely a coincident phenomenon. He also develops the outline of a theory of the way in which the intestinal system may be supposed to exert such specific effect. There will be no need, however, to consider this, if it can be shown that the rejected alternative is the correct one.

The whole theory is based on the relation of the "axial gut." Now what is the axial gut? It is what zoölogists call the anterior ramus of the intestine, and it extends from the base of the pharynx to the head. (See Fig. 1). That is, it is something that can be defined only by its relation to the pharynx and the head. It cannot, therefore, be otherwise than as stated, if regeneration is to take place at all. The head must form in front of it, the pharynx behind it, and to its sides the sides of the body. If it happened otherwise there would be no regeneration, but the development of a monstrosity.

But the author meant more than a mere truism of this sort; he meant to assert that, however much embryonic tissue might form, it would remain undifferentiated until the establishment of an axial gut, which then exercises its all-compelling influence. How far this conclusion is justified by the mere coincidence of the phenomena dealt with we may now pass on to inquire.

In Bardeen's opinion, then, the localization of regenerating organs depends entirely on the prior regeneration of the axial gut. I think that it can be shown that this conclusion is incorrect in some respects. We

have seen what is the criterion of the axial gut in normal individuals; what is the criterion in regenerated parts? Curiously enough, Bardeen does not dwell on this essential point; but I think that one who reads his paper with sufficient care will see that the criterion actually employed is the same here, *i. e.*, relation to the head and pharynx. If this be so, Bardeen has simply argued in a circle. The consideration of one class of cases discussed by Bardeen will serve to show that this has actually been done. "In the case of a very oblique cross-piece (in front of the pharynx) *a lateral branch of the old axial gut may be transformed into an extension of the axial gut.* The head then develops symmetrically around the tip of this, and hence somewhat lateral to the axis of the parent worm."[1] Why in this case does Bardeen regard a certain lateral branch of the original axial gut as transformed into an extension of the latter? For no other reason than that the head appears lateral to the cut end of the original axial gut. There is, in fact, in such cases, no other criterion of the axial gut. The figures illustrating this very case are conclusive in this respect. Regeneration of head and axial gut are really coincident phenomena. I have moreover a large number of observations that show the form of the regenerating gut to be dependent on the form of the new external parts, and *not vice versa.*

The observations recorded in the second of these notes show that the presence of a certain amount of embryonic tissue in front of an unquestionable axial gut does not furnish all the conditions necessary for the regeneration of a head. It was shown, that in Dendrocœlum there is a region extending a certain distance in front of the pharynx, in which regeneration of a head will not take place. A piece including this region after about two days has a certain amount of embryonic tissue on its cut end; it possesses also a certain portion of the original axial gut; yet a head does not form. Other conditions are necessary.

Concerning the localization of the pharynx in regenerating parts, it is held by Bardeen that wherever gut pressure is least, *i. e.*, at the place in the system where waste matters tend to accumulate, there the new pharynx forms. The criterion is not so indefinite here, because in all parts containing any of the three original main rami of the gut, by "place of least pressure" is meant simply the most

[1] *Loc. cit.*, p. 35. The development of the new head lateral to the axis of the parent worm in the case of oblique cuts was noticed first by Morgan and received extensive treatment at his hands.

proximal part in relation to the old pharynx. But I do not see how it is possible to determine the place of least pressure in the regenerating system when no part of the original three main rami are present, until they are again established. But by this time the regeneration of the pharynx is usually begun, so that it is often difficult to say what is the determining factor in phenomena that are so nearly coincident.

But here again comparison of other forms in which the conditions are only slightly different is very instructive. In Phagocata it is very evident that intestinal pressure has nothing at all to do with the regeneration of the lateral pharynges. Reference to Figs. 3, 4, 5, 6, will show that the lateral pharynges develop at the ends of short intestinal branches that are extremely delicate, and in which the intestinal pressure must be higher than anywhere else.

It is at least probable that there is an intimate correlation between regeneration of the intestine and of the pharynx or pharynges. I think that it is also probable that the stimuli inducing pharynx formation proceed from intestinal cells of new formation. But all new portions of the intestine do not induce the formation of new pharynges. There is another determining factor. What is this? It seems to me improbable that Bardeen's answer is in any way correct, because it presupposes a typical form of regeneration of the gut that does not exist. As a matter of fact, the regenerating gut is at first in most cases extremely irregular, and the restoration of its typical form is a matter of secondary regulation, due, I believe, to the form of the body, position of the pharynx and the tendency of the intestinal fluids to flow in the paths of least resistance.

In studies 2, 3, and 4 I have attempted to show the following facts: —

1. The differentiation of exposed embryonic tissue may be dependent on the external stimuli to which such tissue is exposed. The functional correlations of all the parts of a piece capable of regeneration are the internal factors, and the various stimuli from without thus induced in normal sequence are the external factors which determine the location of organs. The case of Dendrocœlum appears to indicate that functional correlation is dependent on the nervous system. The regeneration of a head lateral to the axis of the parent worm in the case of parts cut off obliquely is thus explained, because stimuli which normally would fall upon the head are received by the most advanced part, which is lateral to the original axis in

the case of such an anterior cut surface. The regeneration of a tail is similarly explained.

2. The intestinal system regenerates in relation to the new external parts, and not *vice versa*, as maintained by Bardeen; from which it follows that the location of new parts cannot be due primarily to the form of the gut.

WOODS HOLL, August, 1901.

Differentiation without Cleavage in the Egg of the Annelid Chaetopterus pergamentaceus.

By

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With Plates XXVII and XXVIII.

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Introduction.

The phenomenon of differentiation without cleavage may occur either in fertilized or unfertilized eggs of Chaetopterus. The essential point is briefly this: that, after a short exposure to certain solutions, the eggs, without dividing into cells, may slowly pass through certain well defined phases of differentiation, the yolk accumulating in a dense mass in the interior, and the peripheral cytoplasm becoming vacuolated and ciliated. The ciliated ectoplasm and the yolk-laden endoplasm are analogous to the ectoderm and entoderm of the trochophore, and the phases of differentiation resemble some of the normal processes, though the resulting object can by no stretch of the term be properly called a trochophore. In the case of the unfertilized ova, the action of the solution may be said to induce certain phenomena that would not otherwise appear; and, in the case of the fertilized ova, to suppress certain conditions that would otherwise arise. But the nature of the phenomenon is essentially the same in both cases.

As MEAD has shown ('97), Chaetopterus is a very favorable form for work, because one may remove any desired number of the sexual segments without apparent harm to the individual; the remaining sexual segments may be removed one or a few at a time, as desired; so that, in the breeding season, one has perfect control of his material. During the entire breeding season each sexual segment of the females contains hundreds of ripe ova, in which the germinal vesicle remains

intact, until the eggs come into the sea-water, when the germinal vesicle breaks down, and the first maturation spindle is formed, and moves to the periphery of the egg. The nine chromosomes pass into an equatorial plate, and, unless fertilization is accomplished, this condition remains unchanged for an indefinite period. Fertilization causes the rapid formation of the polar bodies; and the entire development follows. As MEAD first demonstrated ('98, 2), the addition of a small quantity of potassium chloride (KCl) to the sea-water containing the eggs brings about the formation of the polar bodies; not only this, but some farther steps preparatory to the first cleavage may follow; e. g. the formation of the yolk-lobe. But MEAD did not observe the process to proceed farther, although he gave an interesting account of the accompanying cytological phenomena.

Later LOEB ('01) maintained that KCl, as well as certain other salts, in the proper degree of concentration, may bring about development of the unfertilized eggs at least as far as the trochophore stage. The cleavage under these circumstances is, according to LOEB, extremely irregular; and, as he believed the ciliated bodies resulting to be trochophores, he concluded that the cell-lineage is probably a secondary element in the development. Another striking suggestion made by LOEB is contained in the following quotation: »I have often wondered whether it was possible for the unfertilized K-eggs to reach the trochophore stage without any visible external signs of cleavage« (l. c. page 443). He thus raised the whole question of the significance of the cleavage of the egg, and of the rôle of cell-division in development. Having been interested for some time in this problem, I determined to repeat and to modify LOEB's experiments with a view to careful morphological study both of the process of cleavage and of the ciliated structures formed under these conditions. The results have proved unexpectedly interesting.

The experiments were carried on at the Marine Biological Laboratory of Wood's Holl in the summer of 1901, as follows: The females were isolated and kept separate from the males in running sea-water for at least a day before using. But, even then, as I had found in previous seasons, if the eggs were removed directly a few would develope, being fertilized by spermatozoa contained in the slime covering the body. If, however, the females were first washed for a minute or two in running fresh water, before using, the adherent spermatozoa were killed. This precaution was invariably taken in the experiments with unfertilized eggs; and in some cases

the female was twice thus washed in fresh water, once about twelve hours before using, and again immediately before operating; but this extra precaution was unnecessary. In no case, in the experiments on unfertilized eggs, was a single egg of the control series observed to develope.

The sexual segments were removed, and cut open in sea-water; the eggs were then teased out, and separated from fragments of tissue as far as possible. They were then divided among certain solutions, a list of which is given below; but some were, in each case, retained in sea-water for control. After being in the solution for from half an hour to an hour, the eggs were returned to sea-water.

Twenty-six series of observations were carried through, five of which were with fertilized eggs. In addition, the normal development was studied a number of times. From sixteen of these series material was preserved at frequent intervals, and both whole mounts and sections studied.

In explanation of the following table, it may be said, that the $2^1/_2$ N solutions were made up as follows: two and one half times the molecular weight of the salt in grammes was dissolved in a sufficient amount of distilled water, enough being subsequently added to bring the total volume of the solution to 1000 ccm. The numbers of the experiments should be understood thus: the lots of eggs used, twelve in all, were numbered consecutively, 1 to 12. Each lot of eggs was usually divided among several solutions, which received, in each case, a second number; thus 6.1 is the portion of lot of eggs 6, that was put in 90 parts of sea-water plus 10 $2^1/_2$ NKCl, 6.2 another portion of the same lot put in 95 parts of sea-water plus 5, $2^1/_2$ NKCl.

Table of experiments on the eggs of Chaetopterus, 1901.

	Solutions employed	Numbers of the experiments	
		Unfertilized	Fertilized
1	97 s. w. + 3 $2^1/_2$ NKCl	5.1; 6.3; 8.1; 10.1	—
2	95 s. w. + 5 $2^1/_2$ NKCl	6.2; 8.2	—
3	93 s. w. + 7 $2^1/_2$ NKCl	8.3; 9.1; 10.2	—
4	90 s. w. + 10 $2^1/_2$ NKCl	6.1; 7.1; 8.4	12.1; 12.2; 12.3
5	85 s. w. + 15 $2^1/_2$ NKCl	7.2; 11.1	12.4
6	80 s. w. + 20 $2^1/_2$ NKCl	11.2	—
7	90 s. w. + 10 $2^1/_2$ $NCaCl_2$	10.3	—
8	85 s. w. + 5 $2^1/_2$ NKCl + 10 $2^1/_2$ $NCaCl_2$	8.6; 9.2; 11.3	—
9	80 s. w. + 10 $2^1/_2$ NKCl + 10 $2^1/_2$ $NCaCl_2$	11.4	12.5

The formulae should be read thus: Number one, 97 parts of sea-water plus 3 parts of a $2^1/_2$ normal solution of KCl etc.

To follow the order of the experiments would make the description unnecessarily long. I will, therefore, first describe some of the ciliated parts, as they appeared in the living condition, and compare them with the trochophore; then the finer structure will be examined with a view to elucidating their morphology. In the second part I will take up the development of the ciliated structures; in the third the phenomenon of fusion of eggs; and in the fourth a brief discussion of results.

1. Description and Morphology of the Ciliated Structures.

A. From unfertilized ova.

We will describe here only the eggs that have been in solutions 1 to 6, that is the KCl series. The effect of these various solutions is very similar as far as the end-result is concerned, except that the stronger solutions seem usually to cause the production of a greater number of ciliated parts, and the two strongest, 5 and 6, to cause a greater degree of fragmentation of the eggs.

About 24 hours after the beginning of the experiments, in successful cases, the cultures are found to be swarming with ciliated structures, some only feebly stirring or stirring not at all, others moving with varying degrees of rapidity, and a few whirling rapidly. But most keep near the bottom of the dish and do not rise like trochophores. Their movements, moreover, are vague and indirected, entirely unlike the ordered swimming of the trochophores; and they may be of all sizes from small fragments, one fourth or even less of the size of the egg, up to fusions of four or five eggs.

Fig. 1 shows one of the best developed of the thousands of ciliated parts that came under my eye; it is from series 8.2, and was drawn with the camera in the living condition about 24 hours after the beginning of the experiment. The part is radially symmetrically around its long axis, and a constriction at right angles to this axis divides it into a smaller and a larger hemisphere. The yolk (*y*) is aggregated in a dense mass in the latter; a row of large vacuoles (*v*) completely surrounds the body just back of the constriction; the protoplasm of the smaller hemisphere is very dense and granular; the cilia were unusually strong and active, and arranged with great regularity. No cell-structure could be detected in

the living object. This structure possesses a certain undeniable resemblance to a trochophore: if the smaller hemisphere be compared to the pre-trochal, and the larger to the post-trochal region, the large vacuoles occupy approximately the position of the prototroch. It is interesting to note that a similar girdle of large vacuoles is found in this position in the trochophore (Fig. 8). Continuing the comparison, we may note that the aggregation of yolk is in a similar position to the gut of the trochophore. It is, in fact, probable that the smaller hemisphere is homologous to the pre-trochal region, and the larger hemisphere to the post-trochal region of the trochophore. By this I mean that they are probably derived from homologous regions of the egg. However the structure just described was derived from an unsegmented egg. Several pieces similar to the above were seen in the cultures; but they were rare, though, I think, of typical occurrence.

The condition shown in Fig. 2, from series 8.1, 23 hours old, is more common. In this case there is only a general ovoidal symmetry; the vacuoles are distributed rather unevenly; the layer of ectoplasm is wide, and the yolk aggregated in the center.

It frequently happens that the aggregation of yolk becomes separated from the bounding ectoplasm by a cavity. Such a condition is shown in Fig. 3, from series 8.1, 23 hours old. The ectoplasm in this case is full of small vacuoles similar to those of the trochophore.

Fig. 4, from series 7.1, 24 hours old, shows the ciliated product of several fused eggs. In this case there is an enormous central cavity containing large yolk-balls. The thick walls are almost entirely free of yolk, and the ectoplasm is much vacuolated. Many ciliated parts derived from egg-fragments are found; one of these, from series 7.1, 24 hours old, is shown in Fig. 5.

In addition to such uniformly ciliated parts, the same cultures contained many with only a partial covering of cilia; and the majority were entirely devoid of them, but showed by their clear appearance, and frequently by slight contractions, that they were living. I often wondered why these had not produced cilia; and finally found a possible explanation. In Fig. 6 is shown a piece from series 8.1, 23 hours old, entirely devoid of cilia, except a few that have developed around the neck of a small protrusion of naked protoplasm, that has flowed out through a small opening in the ectoplasm. In Fig. 7, from series 7.1, 24 hours old, is shown a similar case. Here

two pieces without cilia are united by slender necks to a naked ball of protoplasm, which has evidently flowed out through a rupture; the common naked ball is covered with long, coarse cilia, that moved slowly. These and similar cases suggest, that the cytoplasm may in most cases have reached a stage of development favorable to the formation of cilia; but that this is inhibited in many cases by secondary conditions, the nature of which is obscure. In another way these figures are instructive, as showing the absurdity of interpreting every ciliated thing in such cultures as a trochophore.

Fig. 8 is a drawing from life, with the camera, of a trochophore of Chaetopterus, about 24 hours old, reared from a fertilized egg. The form is, in general, pear-shaped, the broad end being anterior. The prototroch is recognizable from its longer cilia, but the ciliation is otherwise nearly uniform, except for a short naked zone a little distance posterior to the prototroch. The apical flagellum is extremely long, and composed of a number of agglutinated elements, often seen separated. The ectoderm is riddled with small vacuoles, and a ring of large vacuoles lies beneath the prototroch. The gut and mouth are clearly seen behind the prototroch.

It is thus evident that the ciliated structures, even in the living condition are very little like trochophores.

The nuclear conditions of the ciliated structures give positive proof that we are not dealing even with abnormal trochophores, and show what is the true interpretation. The nuclei can be studied only after staining. For our present purpose the figures of entire mounts will suffice (Figs. 12, 13, 14). These show not a multicellular structure, nor a syncytium with scattered nuclei, but an undivided, though locally differentiated mass of cytoplasm, containing usually, except in case of fusions, a single, large, diffusely staining nuclear area. This area may have a somewhat homogeneous appearance in entire mounts, or it may be more or less dotted with granules of chromatin. Into the minute structure, however, it is not my intention to enter at this place. The yolk is not so conspicuous in the entire mounts as in the living material.

In some ciliated parts I have been unable to distinguish nuclear material with certainty in the entire mounts; but I am not prepared to state positively, that a differentiated nucleus was wanting in these cases. However no multicellular ciliated part, nor any, except in the case of fusions, with many nuclei were found in these preparations. The examples shown in Figs. 12, 13 and 14 are typical. Examples

with a single nucleus, as in Fig. 12 are the most common. The specimen shown in Fig. 13 is decidedly unusual, in that it is, apparently, a single egg, in which one cleavage plane has persisted. Fig. 14 is a good example of the condition of masses formed from several fused eggs. The only region in which cleavage has gone beyond two or three products is devoid of cilia, a condition that seems to be typical and significant.

Thus the remarkable fact appears, that, so far as these observations extend, the ciliated structures are usually unsegmented, and characterized by a single, large, diffusely staining nuclear area. I think that my observations are sufficiently extensive to warrant the conclusion that the majority, at least, possess a similar structure. The difficulty in making an all-inclusive assertion to this effect is, that the cilia are usually very short, and not easily seen in the preparations, while the nuclei can not be seen in the living material; so that it is difficult to correlate the observations on the living and the stained material. Cleavage certainly takes place to a certain extent in all of the cultures, but study of the preparations has led me to the conclusion, that the process of cleavage rarely extends very far, and that such segmented ova are much less likely to become ciliated than entirely unsegmented parts.

It will be asked, are trochophores never formed in the cultures of unfertilized eggs? Only two trochophores have come under my observation in such cultures. One was seen in the living condition; the other was found in one of the preparations, and was a very young stage. I think it quite possible, therefore, that normal development of the unfertilized eggs may take place, though one cannot avoid the suspicion, that those actually observed may have come from eggs accidentally fertilized.

B. Experiments on fertilized eggs.

Experiments were made on only a single lot of fertilized eggs, but the results were, in some ways, very striking and instructive, when viewed in the light of the results of the unfertilized series.

Unsegmented ciliated parts, similar in all respects to those produced from unfertilized eggs, were formed in 12.1, 12.2, 12.4 and 12.5. In the last case alone were there no trochophores. The important point is thus established, that differentiation without cleavage may occur in fertilized, as well as in unfertilized eggs. A culture, like 12.1, that contains both trochophores and the ciliated structures

Experiments 12.1 to 12.5; eggs of Chaetopterus fertilized 1 43 p. m. July 29, 1901.

Solutions employed	Eggs left in solution	Examination 18—19 hours later
1. 90 s. w. + 10 2½ NKCl	From 1 48 to 2 48 p. m.	Some abnormal trochophores; many undivided ciliated parts; most on bottom.
2. 90 s. w. + 10 2½ NKCl	From 2 10 p. m. (first polar body just forming) to 3 10 p. m.	Intermediate between 1 and 3.
3. 90 s. w. + 10 2½ NKCl	From 2 20 p. m. (second polar body just forming) to 3 20 p. m.	Like control; all normal.
4. 85 s. w. + 15 2½ NKCl	From 2 10 p. m. (first polar body just forming) to 3 10 p. m.	Few abnormal trochophores; some ciliated undivided parts.
5. 80 s. w. + 10 2½ NKCl + 10 2½ $NCaCl_2$	From 2 20 p. m. (second polar body just forming) to 3 20 p. m.	No cleavage; no trochophores; fusion masses with ciliated areas common.
6. Control	—	Fine lot of trochophores strewn through water like motes in a sun-beam.

proceeding from unsegmented eggs, brings out very sharply the differences between them; by no possibility can the two types be confused.

In experiment 12.1 there were very few trochophores, mostly abnormal, and a great many of the unsegmented ciliated parts; 12.2 was intermediate in these respects between 12.1 and 12.3. In 12.3 only trochophores were formed; and these were as many and as normal as in the control. These three were portions of the same lot of eggs, and were put in the same solution for the same length of time; the only difference being the stage during which they were exposed. 12.1, the culture most injured by the solution, was put in the solution five minutes after the admixture of the spermatozoa; 12.2, which was injured relatively little, was put in as the first polar body was being formed in the majority of the eggs; 12.3 was put in as the second polar body was forming, and in this case no injury to the eggs resulted. It is plain, therefore, that before maturation and the union of the germ-nuclei, the eggs are in a peculiarly susceptible (weak) condition; or, in other words, that, during maturation and

fertilization, changes are produced in the cytoplasm, that render it much more resistant to the action of the solution.

This observation may be compared to those of MORGAN ('95) and DRIESCH (1900, page 414) showing a change in the physical properties of the egg-cytoplasm immediately after fertilization in the echinid ovum. In the case of Chaetopterus both maturation and fertilization are going on at the same time; so that one can not decide directly to which of these processes to ascribe the increased power of resistance. But in the experiments of DRIESCH and MORGAN this difficulty does not exist; for the maturation of the eggs is completed, before the entrance of the spermatozoon. It is probable, therefore, that the increased power of resistance is the effect of the spermatozoon, and it is important to note that this effect is produced in full long before the union of the egg- and sperm-nucleus. The basis of resistance in this case would seem to be loss of water on the part of the egg, and resulting higher osmotic index of the cytoplasm, rendering it less susceptible to injurious action of solutions of greater density that the sea-water. These results, if confirmed and extended, would strongly confirm LOEB's theory (LOEB, 1900), that loss of water is a primary effect of the entrance of the spermatozoon, and that this is the stimulus to division of the egg.

2. The Development of the Ciliated Structures.

A. The living egg.

The study of the development of the ciliated parts is complicated by the fact, that, even in the most successful cultures, not more than one half or one third of the eggs becomes ciliated, although nearly all remain alive until after the beginning of the period of ciliation. The cultures usually exhibit a great variety of conditions during the first seven or eight hours, and, as time was wanting to carry out isolation experiments, it is difficult to ascertain with certainty which conditions are those leading to ciliation. Study of the preparations, however, shows that the apparent diversity in the living cultures is in the main superficial, being due, in large part, to the greater or less development of pseudopodia, and the occurrence to a greater or less extent of pseudo-cleavage.

MEAD ('97 and '98) has given a very full and accurate description of the maturation, fertilization and cleavage of this egg. The vitelline membrane is thick, transparent and closely adherent to the egg. It

is apparently quite soft for some time after the egg is deposited, because the polar bodies often appear embedded in it. The boundary between the membrane and the likewise transparent ectoplasm is difficult to fix with certainty. During the mitoses of maturation and cleavage the membrane is temporarily thrown into a great number of fine folds, giving it a wrinkled appearance. The appearance of the folds coincides with that of waves in the ectoplasm, so the folds pass slowly wave-like over the membrane in a regular direction and sequence. The amount and arrangement of the yolk in the unsegmented egg is shown in Fig. 22. The cleavage is strictly determinate with a definite cell-lineage (see MEAD, '97).

The rate of development is very rapid. The following table offers comparison between the rate of the normal development and the modified development without cleavage.

		First polar body	Second polar body	Yolk-lobe	Cilia
Normal	Fertilization	17—18 min.	25—26 min.	43—45 min.	6—7 hrs.
Unfertilized	Weak solution (no's 1 and 2)	36 min.	53 min.	1½—2 hrs.	8—9 hrs.
Unfertilized	Strong solution (no's 4, 5, 6 etc.)	not formed	not formed	not formed	8—9 hrs.

From this table it appears: 1) that the formation of cilia takes about two hours longer in the potassium than in the normal development; 2) that the delay is disproportionately great in the maturation stages, the formation of the polar globules and the yolk-lobe taking about twice the normal period; 3) that strong solutions completely inhibit the formation of the polar globules, but that this does not interfere with the subsequent development, which goes on as rapidly after action of the stronger as the weaker solutions, and usually in a greater proportion of the eggs.

A direct effect of the potassium chloride solutions, and still more of those containing also calcium chloride, is the destruction of the egg-membrane. This takes place most rapidly and completely in the stronger solutions. The membrane first becomes inflated, and stands off from the egg in various places like blisters. Later it breaks up into small parts, that float away. The disappearance of the membrane

is an important factor in what follows, for there is now nothing to confine the naked protoplasm.

The following is a description, taken from my notes, of the development of a single culture. July 24, 1901; experiment 9.1. Some unfertilized eggs were put in 93 parts of sea-water plus 7 parts of a $2^1/_2$ N solution of KCl at 9 a. m. They were put back in sea-water at 10 a. m. (one hour). At 9 55 a. m. the eggs have become quite irregular on the surface, but no polar bodies are formed. At 10 32 the membrane is becoming greatly expanded in many eggs; some eggs are indented, others lobed; no polar bodies. At 11 13 (2 hours and 13 minutes) many of the eggs have a small pseudopodial area; sometimes a single long pseudopodium is formed in this region. At 11 25 nearly all of the eggs have such pseudopodia, and a very few are apparent in the two-celled stage. (However preparations made at this time show that, as a rule, this is division only of the cytoplasm, all of the nuclear material being in one cell of the two; cf. Figs. 15 and 16.) At 12 noon the membrane is ruptured in many eggs. Over the pseudopodial area it is invariably much distended, and little balls of protoplasm loaded with yolk-granules are often separated off, looking like micromeres. In other cases the area between the surface of the egg and the distended membrane is bridged by numerous extremely delicate strands of hyaline protoplasm, anastomosing in numerous protoplasmic islands, recalling the appearance of the pseudopodia of Foraminifera. At 1 30 p. m. the activity of the eggs is at its greatest height; Figs. 9 *A* and 9 *B*, of groups of eggs as they lay in the field of the microscope, will show the various forms assumed by the eggs in their active amoeboid movements. Pseudo-cleavage, due to the separation of pseudopodia is very common; so also is the reverse process, the running together of separated parts [1]). At 5 20 p. m. amoeboid activity still continues, and the aggregation of the yolk, that has been slowly progressing from the start, is very manifest. At 5 45 p. m. the first cilia made their appearance.

The description of another culture (series 12.5) will serve to bring out some of the variations. These eggs were fertilized at 1 43 p. m., July 29, 1901; and at 2 20 p. m., as the second polar body was forming in most, they were transfered to a solution made up of 80 parts of

[1]) I am of the opinion, that non-nucleated cells sooner or later invariably fuse with the main mass, which would explain the extreme rarity of ciliated structures with persistent cleavage-planes. In other words cell-walls are retained only between nucleated cells.

sea-water, 10 of $2^1/_2$ N KCl and 10 parts of $2^1/_2$ N $CaCl_2$. At 3 20 p. m. they were put back in sea-water. The solution inhibited the cleavage, so that when they were put back into sea-water none were divided. The formation of pseudopodia on practically of them began at once. At first knob-like pseudopodia were formed in nearly every egg from all parts of the surface (Fig. 10 *A*). At 3 55 there was a strongly marked tendency in some for the pseudopodia to become reduced in number; Fig. 10 *B* shows a case in which a single long tongue-like pseudopodium was carrying off the larger portion of the egg; by 4 p. m. this was entirely retracted, and the egg was round again. In another case (Fig. 10 *C*) part of the long pseudopodium was cut off as a sphere. At 4 13 the display of amoeboid activity was amazing (Fig. 10 *D*); the eggs crept together and very extensive fusions took place. At 4 p. m. few of the eggs remained separate, and a small proportion of these had divided once. Otherwise no cleavage took place. The next morning the culture was full of ciliated parts.

In general it may be said, that the period preceding ciliation is characterized always by amoeboid activity. In some of the cultures a small proportion of the eggs appear in the living condition to be undergoing regular cleavage, which, in a small fraction of these cases again, seems to approximate to the normal. I am not sure that these ever proceed to the formation of cilia; usually they break up in later stages. Most of the segmenting eggs divide very irregularly, and in many cases apparent cells were seen to fuse with the main mass. So that it was often impossible to distinguish between cell-formation and amoeboid protrusions.

The amoeboid phase lasts for some hours, and still persists with diminished intensity in some, after the beginning of ciliation in others. Practically every intermediate condition between one egg, that is simply slightly amoeboid, a second with profusion of pseudopodia, a third with irregular cleavage and a fourth with regular cleavage may be found in the same or various cultures. This fact indicates that amoeboid movements of the cytoplasm may play an important role in normal cell-division, in which case the movements are checked and regulated by the egg-membrane and the nuclear conditions. In Chaetopterus the extent of the movements is normally regulated by the thick, closely adherent membrane, which renders them inconspicuous; but the waves, that may be observed to pass over the membrane in regular sequence and direction, are indicative of internal commotion.

B. The preparations.

The nuclear conditions during the amoeboid and cleavage stages are extremely interesting and deserving of more careful attention than I have yet been able to give them. Description of the series 9.1 will be given first. This should be compared with the account of the living material. As already stated, no polar bodies were formed in this series, but the germinal vesicle broke down and the usual reduced number (9) of chromosomes was formed. These accumulated immediately beneath the periphery at the animal pole (presumably). Radiations at first extend from this group into the cytoplasm; but these are soon lost, and the chromosomes move close together. They then appear imbedded in a circular, sharply defined, non-staining, granular area (Fig. 22). By degrees they break up into smaller granules within this area (Fig. 23). The farther observable changes in most eggs of this series consist simply in slow growth of the nuclear area to gigantic proportions (Figs. 24 and 25). There is a little direct evidence that nuclear material migrates from the area into the cytoplasm (cf. Fig. 25).

There can be little doubt that this is the usual behaviour of the nucleus in the unfertilized eggs, that become ciliated. But many different modifications may be observed in the same or in different experiments. Some of these are illustrated. Figs. 15 and 16 are two eggs of series 10.1, four hours after the beginning of the experiment; they are apparently in the two-celled stage and they represent the typical condition of the eggs of this series and stage. But all of the nuclear material is confined to one of the cells. The position of the nuclear material within this cell is very variable; so also is its condition. The chromosomes may still be separate or fused in a fairly homogeneous nuclear area. The direction of the cleavage plane is, however, normal, i. e. it passes in all cases observed through or near the animal pole, as determined by the polar globules (that were formed in this series).

The phenomenon of cytoplasmic without nuclear division is very common in all the series of unfertilized eggs. It is well illustrated by Figs. 18 and 19 (series 11.2), and it occurs equally well marked in series 9.1 and 10.1. The strength of the solution employed, within the limits of the experiments, makes apparently but little difference in this respect. I am not able to decide positively whether non-nucleated cells divide; but the preparations indicate that they may.

The growing nucleus is, in some cases, caught by the cytoplasmic constriction, and so divided mechanically, as in Fig. 17; the initiative is clearly on the part of the cytoplasm. Similar conditions are shown in Figs. 18 and 19. Figs. 20 (series 11.1) and 21 (series 11.3) show fairly regular cleavages, the most perfect on my slides, in which the cytoplasmic and nuclear divisions are synchronous.

Figs. 26 and 27 from series 12.5 show two fertilized eggs. Fig. 27 shows especially well the amoeboid form fixed by the reagents. In the former figure there is a multipolar spindle and in the latter numerous scattered particles of chromatin. The chromosomes are in process of breaking up, and the granules are scattering throughout the cytoplasm, the larger portions, however, remaining in the center. It is not possible to believe that the scattered particles are ever entirely gathered in again. A great part of the granules must be lost in the cytoplasm, and never form part of the nuclear area, that is seen later in each egg. In this case there can be little doubt that diffusion of nuclear material throughout the cytoplasm takes place. The ultimate effect of this is, of course, problematical; but it may be significant that this experiment was one of the most successful, in some ways the most successful, of the entire series.

Even the smallest discernible particles of chromatin exercise, apparently, a liquefying effect on the cytoplasm, and thus appear to lie in vacuoles (Fig. 27), a condition analogous to the return of the nucleus to its resting condition.

3. Fusion of Eggs.

In his experiments on parthenogenesis in Chaetopterus ('01) LOEB observed the frequent partial or complete fusion of two to five eggs, and interpreted the resulting ciliated structures in two ways: those, in which the constituent yolk-masses had fused into one, were, according to LOEB, giant trochophores; but the majority, in which the yolk-masses remained separate, were compound, i. e. double, triple, quadruple, etc. monsters. In all of my experiments such fusions were common; in the majority of such cases the yolk-masses of the constituent ova remained separate, and the latter exhibited relations similar to the constituent cells of a tissue. Fig. 4 shows, however, a fusion-product, in which the limits of the constituent ova have disappeared. Fig. 14 exhibits an intermediate condition.

The fusion is undoubtedly rendered possible by the destruction of the egg-membrane. In the amoeboid stage the naked masses of protoplasm have a strong tendency to adhere, whenever they come in contact.

Upon adding calcium chloride, in order to test its effect upon the differentiations already observed, I was greatly surprized to find, that the tendency to fusion was increased many fold. Without attempting to give an explanation of the fusion phenomena, I may say, that the remarkable effectiveness of calcium in this respect seems to be due, first to an earlier and more complete destruction of the egg-membrane, and second to a heightening of the amoeboid activity.

I will describe in this connection two sets of experiments, the first with unfertilized and the second with fertilized eggs. In experiment 8.6 the unfertilized eggs were put in the following solution: 85 parts of sea-water, plus 5 parts of a $2^1/_2$ N KCl solution, plus 10 parts of a $2^1/_2$ N $CaCl_2$ solution. At the end of an hour they were returned to normal sea-water; at this time the eggs were entirely naked. Amoeboid movements began at once, and in two hours isolated eggs were rare, the great majority being agglutinated in groups of from two to many. About three hours later, i. e. about five hours after the transfer to sea-water, the majority of the eggs were in large masses of 20—40 or more. The amoeboid movements were very marked at this time, and the contour of the composites varied greatly with this. An hour or so later numerous, usually non-nucleated, micromeres were forming at various places on the surface. These were formed as a rule by the separation of small pseudopodia.

Farther growth of the large fusion-masses took place in the next two or three hours, and about nine hours after the transfer to sea-water cilia began to appear over restricted areas. Fig. 11 is an outline of a camera drawing of one of the largest fusion masses under a low magnification. I estimated that about one hundred eggs entered into its composition. Cilia were detected only in three regions, as shown in the figure; but, as they would be visible only on free edges seen in profile, it is probable that other ciliated areas actually occured on this mass. Each of the ciliated areas corresponds to a single egg. The next morning, about 23 hours after the beginning of the experiment, the large aggregations were all dissolved, forming a great deal of detritus and liberating a large number of smaller ciliated parts; the persistent fusions in this case were not much more numerous or extensive than in the cultures without calcium.

The eggs of the second calcium series, that I wish to describe (12.5), were fertilized at 1 43 p. m., July 29, 1901, and about 40 minutes later, as the second polar body was forming, they were put in the following solution: 80 parts of sea-water, plus 10 parts of $2^1/_2$ NKCL, plus 10 parts of $2^1/_2$ $NCaCl_2$ solution. Twenty minutes later the membranes were enormously expanded, and often ruptured, and pseudopodia were very pronounced. At 3 20 p. m. the eggs were put back into sea-water. At 3 40 every egg was studded with numerous hyaline, blunt pseudopodia (Fig. 10*A*); at about 3 50 p. m. there was a marked tendency towards the formation of relatively few long tongue-like pseudopodia, that were sometimes withdrawn, and sometimes cut off (Figs. 10*B* and 10*C*). At 4 13 the eggs were forming a veritable wilderness of pseudopodia, and very extensive fusions were taking place (Fig. 10*D*). At 4 30 the fusions were increasing rapidly in extent; but no cleavage was to be seen, or only a very few of the still isolated eggs had divided once. The observations were then interrupted until the next morning. My notes written at 9 45 a. m. read: »The most complete fusions yet obtained Perfectly complete fusions of 2—5 eggs not rare. The largest fusions (20—100 eggs) persist, and have ciliated areas. Otherwise the appearances are identical with the non-fertilized eggs after the same solution; i. e. there is no cleavage, and the aggregation of yolk is the same.« In this case even the largest fusions persisted and did not break up until the death of the material.

Both whole mounts and sections of various stages of these fusion masses were studied. In general these did not yield results different from the unfused ova. The growth of the nuclear material, pseudocleavage, the development of vacuoles, the aggregation of the yolk, the formation of cilia, etc. took place as in the unfused eggs. Usually each egg of the complex developed independently of its neighbors, the relations of the component ova being much like that of the constituent cells of a tissue. Fig. 14, from series 11.3 illustrates this in the case of a small composite.

The chief result of the fusion experiments is thus to support the results obtained from study of the unfused ova, and to demonstrate the correctness of my interpretation of the ciliated parts ... The ciliated areas are not trochophores intercalated in the mass; they do not differ markedly in structure from neighboring unciliated areas. They are differentiated unsegmented eggs with immense nuclei.

A number of interesting problems, to which I hope to return another season, are suggested by these facts; e. g. have the ova an affinity for each other, like ovum and spermatozoon? And is their union due to a species of cytotropism? Or is the fusion purely mechanical, as LOEB has suggested, owing to a certain quality of stickiness, increased perhaps by the action of the calcium; so that eggs that accidently touch stick together? Is there the same attraction between the nuclei of two fused eggs, as between the egg and sperm nuclei? Is it not possible by some variation of the method to induce fusion followed by normal development of the constituent parts or fused whole? The material seems favorable for the extension DRIESCH's interesting results on fusion of ova (1900).

4. General Conclusions.

The formation of these ciliated structures is as truly a process of development as the formation of a trochophore. As a matter of fact, the development in the former case takes a little longer than the normal development; the formation of cilia requiring 8 to 9 hours, instead of only about 6 hours. Cilia can no more form on these unsegmented eggs, until a certain process of development is completed, than they can on the two or four-celled stage in the normal development. This point of view is stated in advance, because it might seem to some biologists, that the formation of cilia in this case is simply a response of the unaltered cytoplasm to a modified environment. The data for disproof of this view are scattered throughout the paper, and I would not trouble to specifically combat it, had it not been suggested more than once in conversation.

The most striking feature of these observations is the independence of phenomena usually so closely related in various ways as to appear necessarily related, at least in their order of succession. Thus the formation of the polar globules usually precedes cleavage and differentiation; but this is not necessary, for both the latter processes may occur without the former. Similarly nuclear division usually immediately precedes division of the cell-body; but under the conditions of these experiments cytoplasmic fission may go on without reference to nuclear division. Segmentation of the egg usually precedes differentiation, but it is shown that differentiation may take place without the process of segmentation. None of these processes appears to be absolutely dependent on any of the others;

but the results indicate, that a certain correlation of them is necessary for normal development.

If cell-division has nothing to do with certain processes of differentiation, it can not be the direct cause of any. But it is important to use the word »differentiation« in a clear sense. As used in this article, the word refers to the development of the specific characters of cells, and not at all to the production of the forms, positions, mutual relations and sizes of organs and parts, nor to any of the phenomena that may also be grouped under the term »correlation«.

It is an established fact that the most fundamental phenomena of correlation depend on the polarity of the ovum, and on other less obvious features of its organization. For instance, the relations of ectoderm and entoderm, and probably also, of bilateral symmetry are thus determined.

The inquiry as to the role of cell-division in development is thus narrowed down. But an answer in general terms is a very difficult matter, and an attempt must be regarded as only provisional.

The process of cell-division, as such, is necessary neither to growth, differentiation, nor the earliest correlations; but it is accessory, in Metazoa, to all three as a localizing factor, often from the earliest stages.

The differentiations observed appear to depend on a progressive series of chemical changes, leading to a composition of the protoplasm, in which a certain stimulus (environment) produces a certain morphological result, or in which the products of metabolism appear directly as morphological entities. The series of chemical changes involves both nucleus and cytoplasm; there is possibly a fermentative or catalytic action of nuclear derivatives on cytoplasmic materials. This idea is not original, and is, moreover, vague. But, if it is in the right direction, it will be of more value as a working hypothesis than a false theory, however definite and elaborate.

5. Summary of Results.

1) After being submitted for about one hour to sea-water containing certain amounts of KCl, unfertilized or fertilized eggs of Chaetopterus may develope certain of the organs of the trochophore without cell-division. The ectoplasm becomes vacuolated like the ectoderm of the trochophore, cilia are formed, and the yolk aggregates in a dense

mass. In some cases it is even possible to homologize the regions of these unsegmented ciliated eggs with the regions of the trochophore.

2) These ciliated eggs usually contain a single, large, diffusely staining nuclear area.

3) The development takes a little longer than that of the trochophore.

4) These differentiations may occur without the formation of the polar bodies.

5) The period preceding these differentiations is especially characterized by active amoeboid movements of the protoplasm. Cleavage usually takes place in some eggs, but rarely goes very far. Pseudo-cleavage, i. e. division of the cytoplasm without division of the nucleus, is very common. The non-nucleated »cells« probably invariably fuse, sooner or later, with the common mass.

6) Amitosis is sometimes effected mechanically through constriction of the nucleus by a cytoplasmic cleavage-plane.

7) Some direct evidence was found, tending to show diffusion of chromatin through the cytoplasm, especially in the case of fertilized ova.

8) Fusion of from two to five eggs is very common in the KCl cultures; usually the fusion is incomplete, the constituent ova being separated by cell-walls; but in some cases it is complete.

9) The addition of a sufficient amount of $CaCl_2$ to the K-containing sea-water greatly increases the tendency to fusion. After being in such a solution, as many as 100 eggs may unite in a common mass. Cilia form on limited areas in the course of from nine to eleven hours on such a mass, which may later break up into smaller fusion-masses or separate ova, or may persist until the death of the material.

10) In the case of fertilized eggs, a solution that inhibits the cleavage almost completely five minutes after fertilization, has relatively little effect 17 minutes after fertilization (the time of formation of the first polar body), and no effect 27 minutes after fertilization (the time of formation of the second polar body). The period of exposure to the solution being the same in the three cases.

The University of Chicago; March 1, 1901.

Zusammenfassung.

1) Nach etwa stundenlangem Aufenthalte in mit KCl versetztem Seewasser können befruchtete und unbefruchtete Eier von Chaetopterus gewisse Organe der Trochophora ohne Zelltheilung entstehen lassen. Das Ektoplasma vacuolisirt sich wie das Ektoderm der Trochophora, es bilden sich Cilien und der Dotter sammelt sich zu einer dichten Masse. In manchen Fällen kann man sogar die einzelnen Regionen dieser unbefruchteten gewimperten Eier mit denen der Trochophora identificiren.

2) Diese gewimperten Eier enthalten gewöhnlich einen einzigen großen, sich diffus färbenden Kernbezirk.

3) Die Entwickelung dauert ein wenig länger als die der Trochophora.

4) Diese Differenzirungen können ohne die Bildung von Richtungskörperchen stattfinden.

5) Die ihnen vorhergehende Periode ist speciell charakterisirt durch lebhafte Protoplasmabewegungen. In einigen Eiern treten Theilungen auf, selten aber in größerer Ausdehnung. Scheinbare Theilungen, nämlich Zellleibtheilungen ohne Kerntheilung, sind sehr häufig. Die kernlosen »Zellen« fließen wahrscheinlich unabänderlich früher oder später mit der gemeinsamen Masse zusammen.

6) Amitosis kommt manchmal auf mechanische Weise durch Zerschnürung des Kerns seitens einer cytoplasmatischen Theilungsplatte zu Stande.

7) Es wurden direkte Beobachtungen gemacht, welche eine Diffusion des Chromatins ins Cytoplasma hinein zeigen könnten, speciell bei befruchteten Eiern.

8) Verschmelzung von zwei bis fünf Eiern tritt in den KCl-Lösungen ganz gewöhnlich auf; gewöhnlich ist die Vereinigung unvollständig, indem die sie bildenden Eier noch durch ihre Zellwände getrennt sind; manchmal ist sie aber auch eine vollständige.

9) Ein genügender Zusatz von Chlorcalcium zu den Chlorkaliumlösungen vermehrt die Neigung zur Verschmelzung stark. Nach dem Aufenthalt in solchen Lösungen können bis zu 100 Eier zu einer gemeinsamen Masse verschmelzen. Im Verlauf von 9—11 Stunden bilden sich an bestimmten Bezirken einer solchen Masse Wimpern aus. Die ganze Masse kann später in kleinere Fusionsaggregate oder auch in getrennte Eier zerfallen oder bis zum Absterben des Materials bestehen bleiben.

10) Bei befruchteten Eiern hat eine Lösung, welche, 5 Minuten nach der Befruchtung angewendet, die Furchung verhindert, bei ihrer Anwendung 17 Minuten nach der Befruchtung nur wenig Wirkung (Zeitpunkt der Entstehung des ersten Polkörperchens) und bleibt wirkungslos 27 Minuten nach derselben (Entstehungszeit des zweiten Richtungskörperchens). Voraussetzung ist dabei gleichlange Einwirkung in allen drei Fällen.

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Description of Figures.

All figures of plate XXVII were drawn with the camera lucida from the living object; those on plate XXVIII are camera drawings from preparations, either mounts of entire eggs or sections.

Reference letters: *c* cavity, *n* nucleus, *p* polar bodies, *v* vacuole, *y* yolk.

Plate XXVII.

Fig. 1. A ciliated part from experiment 8.2, drawn 24 hours after the beginning of the experiment. The unfertilized eggs were put in 95 parts of sea-water, plus 5 parts of $2^1/_2$ N KCl for one hour. Leitz oc. 1, obj. 7.

Fig. 2. From experiment 8.1, 23 hours old; unfertilized; solution employed: 97 parts of sea-water, plus 3 parts of $2^1/_2$ N KCl. Leitz oc. 1, obj. 7.

Fig. 3. Same history as Fig. 2.

Fig. 4. From experiment 7.1, 24 hours old; unfertilized; solution employed: 90 parts of sea-water, plus 10 parts of $2^1/_2$ N KCl. Fusion of three or four eggs. Leitz oc. 1, obj. 7.

Fig. 5. Same history as Fig. 4. Part of an egg with very little yolk. Leitz oc. 1, obj. 7.

Fig. 6. From experiment 8.1; 24 hours old; unfertilized; solution employed: 97 parts of sea-water, plus 3 parts of $2^1/_2$ N KCl. Cilia confined to the edge of the rupture. Leitz oc. 1, obj. 7.

Fig. 7. From experiment 7.1; 22 hours old; unfertilized; solution employed: 90 parts of sea-water plus 10 parts of $2^1/_2$ N KCl. The movements of the long, coarse cilia were very slow. Leitz oc. 1, obj. 7.

Fig. 8. A normal trochophore 24 hours old. Leitz oc. 1, obj. 7.

Fig. 9*A* and 9*B*. Experiment 9.1. Two groups of eggs drawn as they lay in the field of the microscope, $4^1/_2$ hours after the beginning of the experiment, showing the various forms assumed by the eggs at this time. Solution employed: 93 parts of sea-water, plus 7 parts of $2^1/_2$ N KCl. Zeiss oc. 6, Leitz obj. 4. Reduced one half.

Fig. 10. From experiment 12.5; fertilized eggs; solution employed: 80 parts of sea-water, plus 10 parts of $2^1/_2$ N KCl, plus 10 parts of $2^1/_2$ N $CaCl_2$. Leitz oc. 1, obj. 7.

A Typical amoeboid condition 80 minutes after exposure to the solution. The circles in the ectoplasm are yolk-spheres.

B Egg with long pseudopodium; 85 minutes after exposure to the solution. The pseudopodium was retracted five minutes later in this egg.

C Two eggs uniting, one with long pseudopodium; about the same age as *B*. The terminal portion of the pseudopodium became separated a little later.

D Five eggs uniting about two hours after putting in the solution.

Fig. 11. Fused mass of eggs from experiment 8.6; drawn 11 hours after the beginning of the experiment. Solution employed: 85 parts of sea-water, plus 5 parts of $2^1/_2$ N KCl, plus 10 parts of $2^1/_2$ N $CaCl_2$. Cilia were formed on this mass in the three positions indicated. LEITZ oc. 1, obj. 4.

Plate XXVIII.

Fig. 12 to 21 are from preparations of eggs mounted entire. The remaining figures are from sections.

Fig. 12. Experiment 11.1. Unfertilized. From material killed in KLEINENBERG's dilute picro-sulphuric acid; 22 hours old. There is a single large nucleus; the protoplasm is vacuolated and ciliated; the yolk lies near the center. ZEISS oc. 6, obj. 4 mm.

Fig. 13. Experiment 10.2. Unfertilized. From material killed in KLEINENBERG's dilute picro-sulphuric acid; 25 hours old. The nuclear material here is in two parts, and there is one persistant cleavage plane. ZEISS oc. 6, obj. 4 mm.

Fig. 14. Experiment 11.3. Unfertilized. Killed in KLEINENBERG's dilute picro-sulphuric acid; 22 hours old. This represents the product of fusion of at least three eggs. It should be noted that the part in which cell-division has gone the farthest is without cilia. ZEISS oc. 6, obj. 4 mm.

Figs. 15 and 16. From experiment 10.1. Unfertilized. Killed in BOVERI's picro-acetic acid four hours after the beginning of the experiment. These two eggs are typical of a large proportion of apparently segmented eggs; nuclear material confined to one cell. ZEISS oc. 6, obj. 4 mm.

Fig. 17. From experiment 11.2. Unfertilized. Killed in BOVERI's picro-acetic acid, five hours and forty minutes after the beginning of the experiment. The division of the nucleus in this case is seen to be a mechanical effect of the cytoplasmic constriction. ZEISS oc. 6, obj. 4 mm.

Figs. 18 and 19. From experiment 11.2. Unfertilized. Killed in BOVERI's picro-acetic acid. The majority of the cells in these two eggs are without nuclei. ZEISS oc. 6, obj. 4 mm.

Figs. 20 and 21. From experiments 11.1 and 11.3 respectively. Unfertilized. Killed in BOVERI's picro-acetic acid, 5 hours and 40 minutes after the beginning of the experiment. These represent the most perfect cleavages found among the preparations. ZEISS oc. 6, obj. 4 mm.

Fig. 22. From experiment 9.1. Material killed in BOVERI's picro-acetic acid, 1 hour and 14 minutes after the beginning of the experiment. Nine chromosomes were counted in the sections of this egg. ZEISS oc. 6, obj. 2 mm.

Fig. 23. From experiment 9.1. Killed in BOVERI's picro-acetic acid, 1 hour and 40 minutes after the beginning of the experiment. Distinct chromosomes can no longer be seen within the nuclear area, which is already considerably enlarged. ZEISS oc. 6, obj. 2 mm.

Fig. 24. From experiment 9.1. Killed in BOVERI's picro-acetic acid, 3 hours and 17 minutes after the beginning of the experiment. Farther enlargement of the nucleus; partial fusion of the yolk-granules. The separated balls below are derived from isolated pseudopodia. ZEISS oc. 6, obj. 2 mm.

Fig. 25. From experiment 9.1. Killed in BOVERI's picro-acetic acid, 8 hours and 22 minutes after the beginning of the experiment. Farther enlargement of the nucleus; at one place the boundary of the nucleus towards the cytoplasm is lost. The individual yolk-granules are for the most part fused in a common mass. ZEISS oc. 6, obj. 2 mm.

Fig. 26. From series 12.5; fertilized eggs killed in BOVERI's picro-acetic acid, 3 hours after the beginning of the experiment. Irregular multipolar karyokinesis. ZEISS oc. 6, obj. 2 mm.

Fig. 27. The same material as Fig. 26. It shows a later stage than the former. The chromosomes are irregularly scattered about, and some have broken up into granules, which are, apparently, liquefying the surrounding protoplasm. ZEISS oc. 6, obj. 2 mm.

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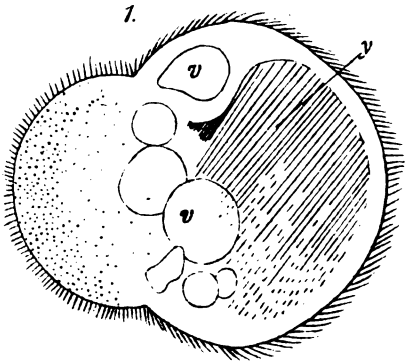

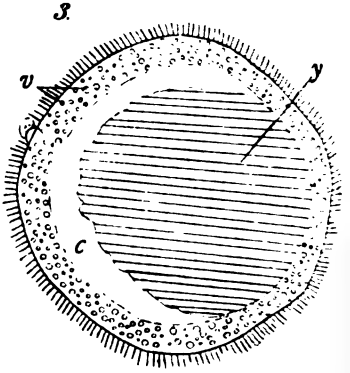

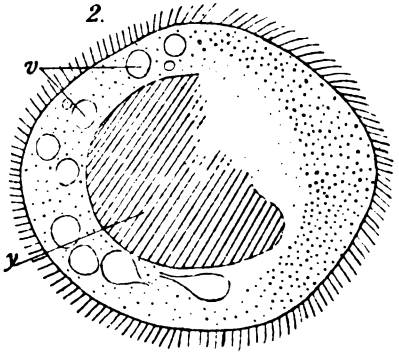

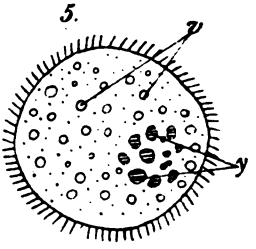

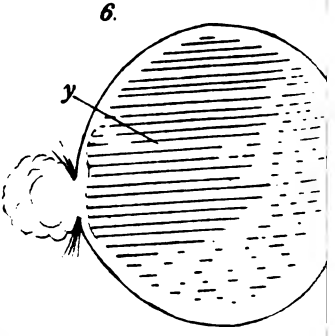

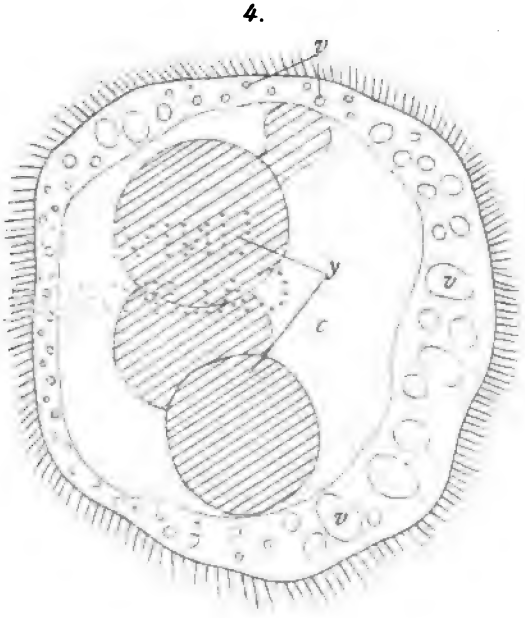

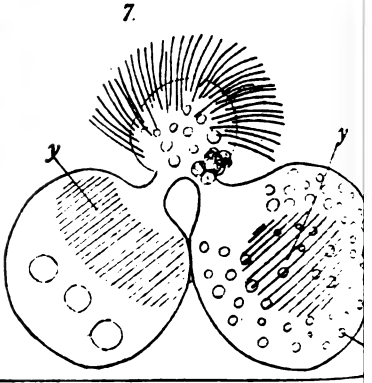

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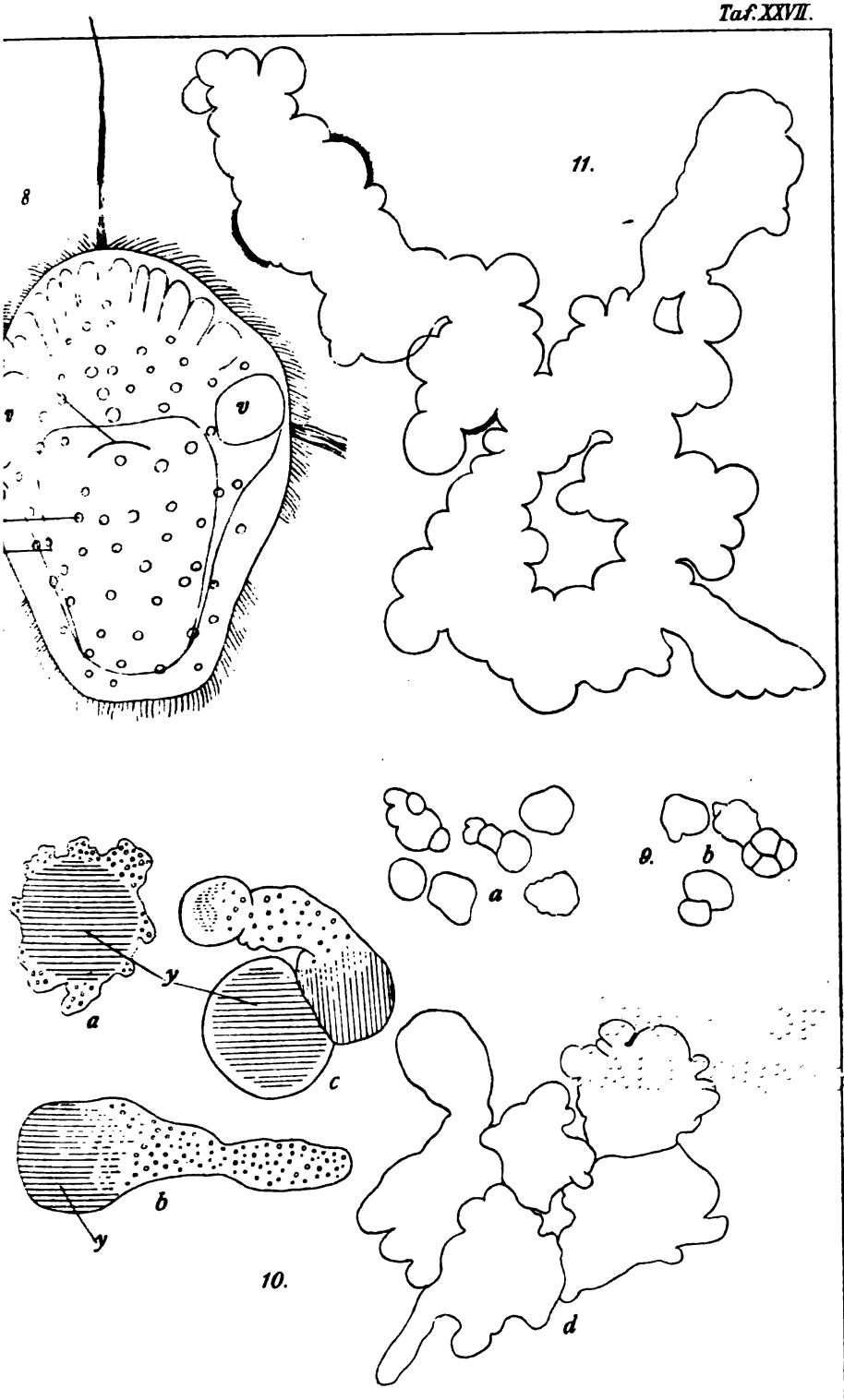

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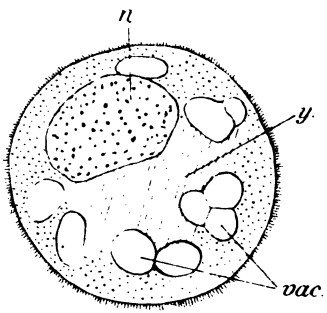

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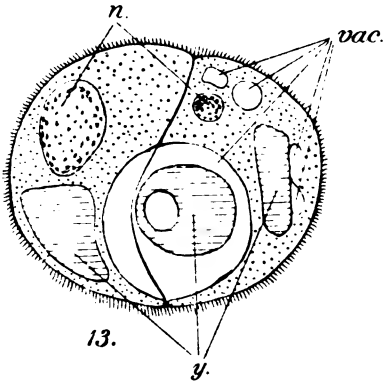

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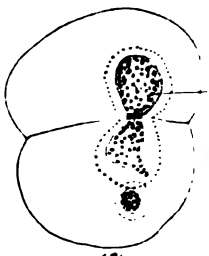

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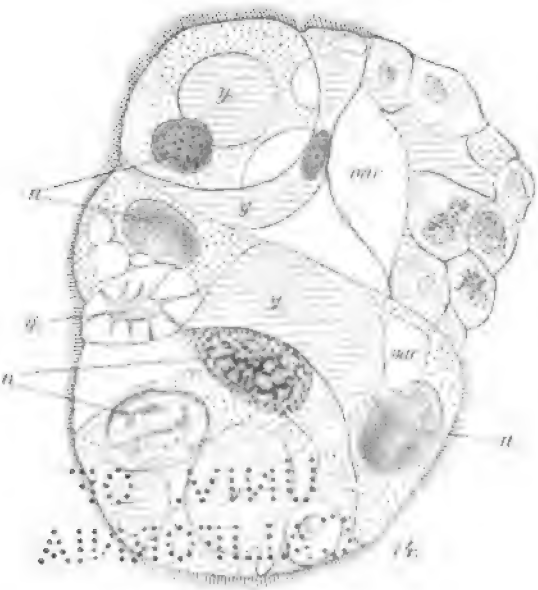

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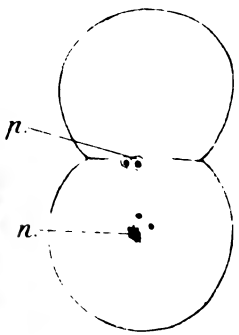

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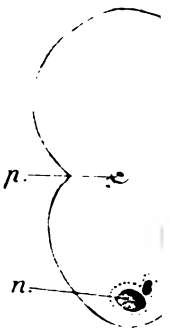

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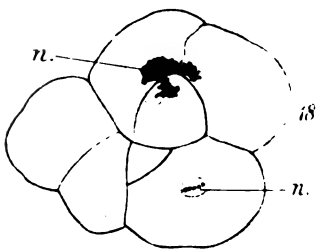

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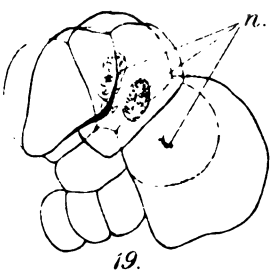

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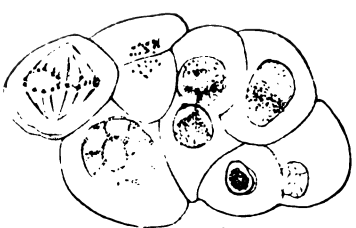

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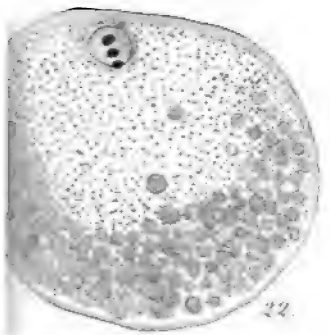

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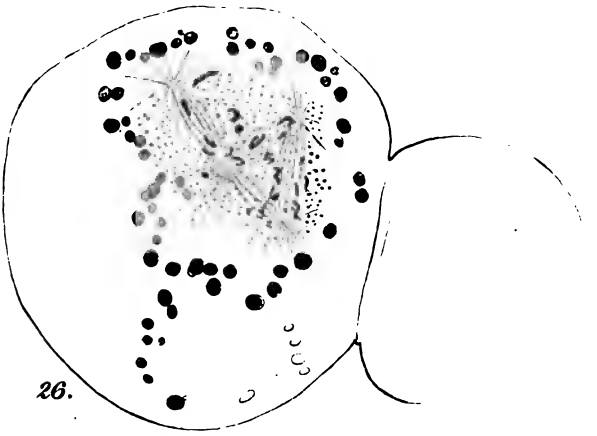

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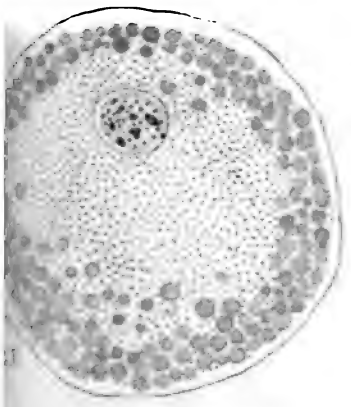

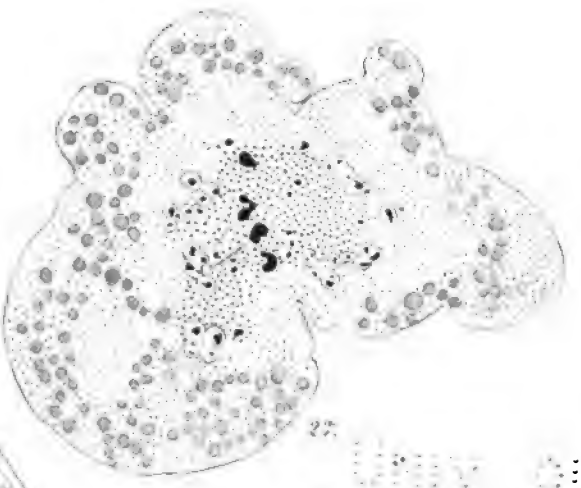

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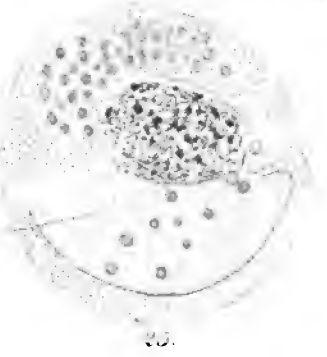

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EXPERIMENTAL STUDIES ON THE DEVELOPMENT OF THE ORGANS IN THE EMBRYO OF THE FOWL (GALLUS DOMESTICUS).

FRANK R. LILLIE.

I. INTRODUCTION.

The results to be described under the above title relate to the morphology, functions and power of regeneration of various embryonic organs, and to the influence that certain embryonic parts exert on the development of others. They represent the application of a particular experimental method, viz., the destruction of definite parts, and study of the subsequent development. Thus the particular organs studied are those most accessible to operation, which form a rather heterogeneous assemblage. Nevertheless, taken as a whole, the results form a contribution to the subject of *correlative differentiation* of organs.

The Principle of Correlative Differentiation in Embryology (*i. e.*, influence of the intraorganic environment in development)[1] is that the rate, degree or mode of differentiation of any embryonic rudiment is dependent on some part or parts of the same organism (individual) external to itself; that is, that component parts of an embryo determine mutually to a greater or lesser extent, their respective lines and grades of differentiation. Much more is meant by this than that any embryonic part can develop only in its normal environment, which offers the prerequisites of its very existence. The principle of correlative differentiation in fact implies a distinction between *a determinative and a non-determinative environment*, and the problem of correlative differentiation is so far resolved when this is ascertained for all the organs (cf. Roux).

Any part, the entire environment of which is non-determinative, is said to develop by *self-differentiation* (Roux).

These two principles do not stand in the relation of rival theories but rather, probably, of coöperative factors in every

[1] Environment may be defined as conditions that influence dynamic processes in protoplasm, and may be divided into extraorganic and intraorganic, the former being external to the individual and the latter within its bounding surfaces.

embryonal differentiation, for any process of self-differentiation of a structure might be analyzable into correlative differentiation of its parts.

For the development of the higher animals at least the extra-organic environment is non-determinative. The development of the ovum as a whole is therefore a process of self-differentiation. But it is usually assumed that it is otherwise with the differentiation of its constituent parts; the extreme view being that each influences the mode of differentiation of all the remainder. From this standpoint the complexity of the correlative processes of differentiation must increase in proportion to the increase in complexity of structure.

Theoretically, at least, the determinative value of correlative differentiation in any case may be (1) absolute, *i. e.*, the mode of development of a part being determined entirely from without itself; (2) partial; (3) wanting, *i. e.*, absolute self-differentiation.

Our present knowledge is enough to exclude the first theoretical possibility. No principle in embryology is better established than that sooner or later the embryo is a mosaic of embryonic rudiments, each of which is to a certain extent self-determining. This mosaic of rudiments may become visible very early, as in those ova exhibiting a definite cell-lineage of organs, or it may appear later. In some cases, at least, the unsegmented ovum itself is a simple mosaic (ovum of ctenophores according to Fischel; ovum of *Unio*, Lillie; ovum of sea-urchins, Boveri; ovum of frog, Roux, Schulze and others). Indeed it is quite probable that all ova are more or less simple mosaics of embryonic rudiments.

Unless, therefore, we wish to beg the entire question we must proceed on the second hypothesis. This is the writer's standpoint, and the problem is to determine as many definite correlations as possible and to investigate their nature.

There is probably no conception in embryology so vague as that of correlative differentiation, as the following citations may serve to show:

Hertwig: "Zelle und Gewebe," II.:

"Die Wechselwirkungen (Correlationen) zwischen den Zellen eines Organismus und ihren Derivaten bilden sich mit dem

Beginn des Entwicklungsprocesses aus, ändern sich von Stufe zu Stufe und compliciren sich in demselben Maasse, als die Entwicklung fortschreitet.

" Im Gegensatz zum Mosaiktheorie von Roux und der keimplasma theorie von Weismann stellt die Theorie der Biogenesis den Grundsatz auf, dass vom ersten Beginn der Entwicklung an die durch Theilung des Eies sich bildenden Zellen beständig in engster Beziehung zu einander stehen, und dass dadurch die Gestaltung des Entwicklungsprocess sehr wesentich mit bestimmt wird. *Die Zellen determiniren sich zu ihrer späteren Eigenart nicht selbst, sondern werden nach Gesetzen die sich aus dem Zusammenwirkung aller Zellen auf den jeweiligen Entwicklungsstufen des Gesammtorganismus ergeben, determinirt.*"

Herbst: " Formative Reize in der Tierischen Ontogenese: "

" Die Aufgabe des zweiten Teiles meiner Abhandlung über die formativen Reize war es also, in der tierischen Ontogenese, abgesehen von der Namhaftmachung jener wenigen Fälle von Gekommen von formativen Reizwirkungen, die von irgend einem Teil des Organismus auf einen oder mehrere andere ausgeübt werden, festzustellen und eventuell die Möglichkeit der vollständigen Auflösung der ganzen Ontogenese in einer Reihe von solchen Induktionserscheinungen nachzuweisen.

"So ist es zum Beispiel zum mindesten ungenau, von der 'weitgehenden Wechselbeziehung' zo sprechen, 'die zwischen allen Teilen eines Organismus auf allen Stadien seiner Entwicklung besteht' (Hertwig: 'Evolution und Epigenesis'); denn das Ektoderm der Echiniden entwickelt sich unabhängig vom Entoderm, und auch abgeschnürte Hautstücke, etc., konnen sich selbständig differenzieren, wie dies das Vorkommen der Teratome beweist (Roux). Die Annahme einer ganz allgemeinen Korrelation zwischen allen Teilen des Organismus auf allen Stadien der Ontogenese ist deshalb ebenso falsch wie jene von der qualitativ ungleichen Kernteilung der Mosaiktheorie."

Most of the real illustrations (*i. e.*, experimentally determined) of this principle must be taken from plants and plant-like animal colonies. One need only glance through Herbst's recent " Formative Reize in der Tierischen Ontogenese " to realize that, so far as egg development is concerned, the application of the prin-

ciple rests very largely on inference, analogy and a few doubtful pathological conditions.

Discussion of this subject belongs, however, to the conclusion rather than to the introduction, and the foregoing remarks are intended only to define the problem.

II. Methods of Operation.

In making the operations one must work as far as possible under antiseptic conditions. Instruments, etc., must be sterilized; this is most readily done by passing the needles, knives, scissors, etc., through a flame immediately before each is used. In spite of all precaution a great many eggs are infected. In my experiments only about 20 per cent. of the eggs remained alive until the time of examination for the results of the experiment, two to five days after the operation. The causes of the mortality in the remaining 80 per cent. are two: (1) Fatal injury of the operation (about 40 per cent. ?); (2) infection with mould or bacteria (about 40 per cent. ?). There is a very noticeable difference between different lots of eggs; some bear operations much more readily than others and are less prone to infection. These differences in the relative powers of resistance of different lots of eggs are due to the relative freshness of the eggs when incubation is begun, and also to the time of year. It is noticeable that in a lot of eggs in which a relatively large proportion, over 50 per cent., fail to develop in the incubator, the percentage of failures in the actual experiments is usually very high.

The method of procedure in my experiments was as follows:

1. The eggs are not turned in the incubator, so that one may be sure of locating the position of the embryo in the unopened egg exactly. The upper side of each egg is marked with a pencil.

2. A small opening is made through the shell and membrane over the embryo.

3. The operation is then made. For cauterization I employ either a needle heated red hot in the flame, or an electric cauterizing needle. The heated needle cools very rapidly, so that the operation must be hastily performed, and it is difficult precisely to delimit the injury. The electric cautery, on the other hand, is apt to give too intense heat. Each method possesses certain advantages.

4. The opening in the egg is closed as follows: A piece of the shell with membrane attached is cut from a corresponding part of another fresh egg, so as to be slightly larger than the opening in the operated egg. This is placed over the opening so as to close it completely; and the albumen adhering to the membrane acts as cement. To ensure perfect closure strips of the egg-membrane are plastered on so as to overlap all edges of the foreign shell. The advantages of this method of closure are that the foreign surfaces are perfectly aseptic if fresh eggs are used, and that the conditions are as nearly like the normal as possible. It is, morover, the simplest and easiest method. This method of closing the opening was first used by Miss Peebles.[1]

III. Experiments on the Amnion and the Production of Anamniote Embryos in the Chick.

A. *The Normal Development of the Amnion.*

The purpose of this section is to give a brief statement of some facts concerning the formation of the amnion before taking up the analysis of the processes by experiment. This is necessary because the facts are at least partly new, and without knowledge of them the mechanics of formation of the amnion cannot be understood. For a recent review of the literature on the whole subject of the amnion in the Sauropsida, see Schauinsland ('02*a* and '02*b*); the latter paper I regret not to have seen.

In the somatopleure on each side of the axis of an early embryo of the chick three zones may be distinguished on the basis of the subsequent differentiation, (*A*) for the body-wall; (*B*) for the amnion; (*C*) for the chorion (serosa) (Fig. 1). It is important to trace the origin of the differentiation between the amnion and serosa on the one hand, and amnion and body-wall on the other, for the conditions that determine the development of the amnion must be antecedent to such differentiation.

1. *The Ectamnion.*—The differentiation of the amniogenous from the choriogenous somatopleure is always preceded by the appearance of a thickening of the ectoderm along the external margin of the former. This thickening, for which I propose the

[1] *Roux's Archiv*, VII., 1898.

name ectamnion, precedes by a little the formation of amniogenous folds in any region, and indeed it induces the origin of the entire system of folds. It has been described by many embryologists at the stages immediately preceding fusion of the limbs of

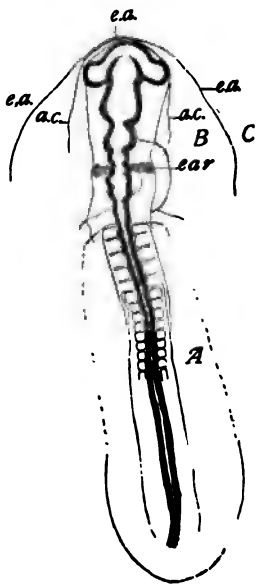

FIG. 1. Embryo of chick with 13 mesoblastic somites. University of Chicago Embryological Collection, No. 555. *e.a.*, ectamnion; *a.c.*, inner margin of amniocardiac vesicles; *A*, region of the somatopleure destined to form the body-wall; *B*, amniogenous somatopleure; *C*, choriogenous somatopleure.

the amnion (cf. Schenk, '71), and it forms the ectodermal seroamniotic connection of Hirota ('94). But no one, so far as I know, has traced it back to its origin and recognized the fact that it is the earliest formed part of the amnion, which is thus primarily ectodermal in the chick, as in Chelonia and some other primitive Sauropsida.

The ectamnion may first be distinguished at about the stage with nine mesoblastic somities, where it appears as a median thickening of the ectoderm in front of the head near the anterior boundary of the proamnion. Along the line of this thickening there is a fusion, between ectoderm and entoderm. The thickening is extended right and left and turns backwards along opposite sides of the head to about the region of the middle of

the heart, gradually becoming more peripheral in position and slowly fading out (Fig. 1). This line represents the junction of the amniogenous and choriogenous somatopleure, and thus corresponds to the angles of the future amniotic folds.

The head of the embryo lies in a depression bounded in front by the ectamnion and on the sides by the amnio-cardiac vesicles of the body cavity, along the inner upper margin of which the ectamnion runs for a short distance. The floor of the depression is the proamnion.

In a stage with 14–15 mesoblastic somites the ectoderm of the proamnion is much more thickened in front of the head, and has

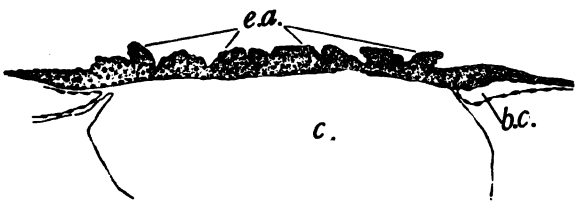

FIG. 2. Transverse section through the anterior angle of the ectamnion, a few sections in front of the tip of the head. 14–15 mesoblastic somites. University of Chicago Embryological Collection, No. 215. *b.c.*, body-cavity; *c.*, large cavity in the entoderm; *e.a.*, ectamnion.

a *villous outer surface* in consequence of irregularity in the thickening[1] (Fig. 2), which may be traced back to the level of the heart, and on one side to its hinder end; there is also a very short ectentodermal fusion beneath the tip of the head. In this series the ectamnion marks the boundary between two distinctly differentiated parts of the extraembryonic somatopleure, the more central of which is the amnion.

In another embryo with fourteen mesoblastic somites, the tip of the head is surrounded by the amnion, and the proamniotic part is represented only by a short median strip extending eight sections back to a point where the limbs of the amnion have not yet closed. The ectamnion is continued only for a short distance along the *angles* of the amniotic fold, and then passes peripher-

[1] In examining the section one receives a strong impression that the irregularities may be due to amœboid movements; but it is not possible to confirm this by actual observations.

ally. How has the head-fold been formed? The great expansion of the body cavity (amniocardiac vesicles) on each side causes an elevation of the anterior angle of the ectamnion and a pocket is formed by fusion of its opposite limbs, which have a strong affinity for each other; fusion proceeds along the median dorsal line so long as the energy of fusion is sufficient to draw the somatopleure up. The head of the embryo is rapidly elongating at this time and slips into the pocket thus formed, being guided in part by the cranial flexure (Fig. 1). It is interesting to note how far the ectodermal thickening stretches ahead of the mesoderm of the fold near the point of closure, and that the apical cells are elongated into pseudopodium-like processes.

The histological differentiation of the amniotic area of the somatopleure from the chorionic portion precedes the elevation of the fold.

This brief inquiry, then, suggests that the order of events in the formation of the head fold of the amnion is:

1. Thickening of the ectoderm on the outer margin of the amniogenous somatopleure, beginning in front of the head of the embryo and extending back on each side (ectamnion).

2. Great expansion of the body cavity on each side opposite the head of the embryo and consequent elevation of the anterior bay of the ectamnion to the level of the dorsal surface of the embryo.

3. Fusion of the right and left limbs of the ectamnion, beginning at the angle, to form a pocket, the head-fold of the amnion.

4. Pushing of the head of the embryo into the fold.

There may be, however, considerable variation in the time of formation of the head-fold. I have, for instance, one series with 17–18 mesoblastic somites (ser. 175), where the head-fold is not yet formed.

Extension of the Ectamnion. — The ectamnion differentiates backward more rapidly than the lateral folds, and always precedes their origin. In the 48-hour stage (21–22 somites) (Fig. 3) the ectamnion from in front has joined that from behind formed in connection with the tail-fold. There is a place, corresponding nearly to the final meeting place of anterior and posterior lateral folds, where it becomes very faint. It would appear

then that behind the tail there is actually a new starting-point for the ectamnion as well as the amniotic folds. The primary position of the ectamnion is near the boundary of the pellucid area; towards the posterior end it bends in very sharply, nearly joining the body wall proper, and terminating in the posterior rudiment.

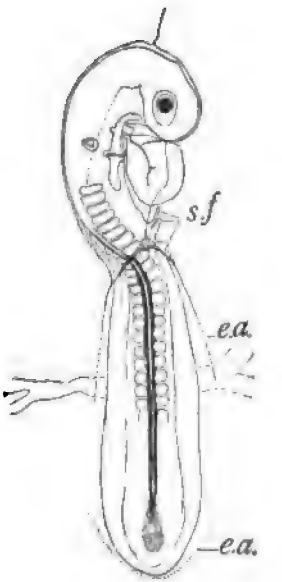

FIG. 3. Embryo of chick with 21 mesoblastic somites. University of Chicago Embryological Collection, No. 99. *e.a.*, ectamnion; *s.f.*, secondary folds of the amnion on the right side. The dotted line continuing *e.a.* represents the continuation of the ectamnion beyond the region of folding. The dotted area at the angle of the folds represents the ectodermal sero-amniotic connection of Hirota.

Origin of the Tail-Fold. — The tail-fold proper arises from an ectodermal thickening lying in a depression just beneath the rudimentary tail-bud. The depression is caused by the enlargement of the body cavity on each side of the middle line. These enlargements may be called the amnio-allantoic enlargements, as they are associated with the formation of the allantois. I would venture the hypothesis that the existence of a *separate* tail-fold of the amnion is associated with the time of development of the allantois, which is represented in the embryo under consideration (1) by a shallow entodermal evagination and (2) a mass of mesoblast.

At the time of formation of the tail-bud a very shallow pocket forms behind it. This owes its origin to the elevation of lateral folds of the somatopleure and progressive fusion beginning at the posterior angle of the ectamnion. The floor of the pocket includes a thick posterior prolongation of the allantoic mesoblast which furnishes a firm floor to the pocket and thus determines the form of the folds.

2. *The Amniotic Folds.* — The subsequent development includes the elevation and fusion of the anterior and posterior lateral folds. The final closure takes place opposite the buds of the hind limbs. The order of events in these processes is as follows:

1. The growth of the amniogenous somatopleure behind the head-fold and in front of the tail-fold.

2. The uprising of the amniotic folds, and their growth in a definite direction around the embryo.

3. The fusion of the right and left folds along the line of the ectamnion in such a way that the external limbs unite to form the chorion, and the internal to form the amnion.

Study of the morphology of these processes suggests the following physiological conclusions:

1. The growth of the amniogenous somatopleure may be a result of the traction exerted in it by the progressive fusion of the folds already formed in front and behind.

2. The uprising of the lateral folds is determined by the head- and tail-folds, the progressive fusion of the right and left ectamnion dragging the amniogenous somatopleure into place.

It remains to test these conclusions by experiments, but before proceeding to a description of these, I wish to describe *the influence of the rotation of the embryo on the amniogenous somatopleure.*

Practically all of the somatopleure of the pellucid area is amniogenous with the exception, naturally, of that part internal to the limiting sulci that forms the body-wall. What effect has the turning of the embryo on its left side on the amniogenous somatopleure? We will suppose that the latter is primitively of equal width on both sides; we will furthermore assume that the somatopleure cannot be drawn in from the vascular area, because it is here attached to the splanchnopleure. (The fusion of the somatopleure and splanchnopleure at the margin of the pellucid

area is shown by the fact that the splanchnopleure is often drawn up with the outer limb of the amniotic fold, making a fold of the splanchnopleure at this place) (Fig. 5). Finally let us assume that the notochord represents approximately the axis of rotation. During the process of rotation the embryo sinks and the lateral limiting sulci become deeper. A direct consequence of the rotation must be therefore a strong tension on the somatopleure belonging to the under (left) side, *a-b*, and practically none on the upper (right) side, *c-d*, (see Fig. 4, *A*, *B*, *C*).

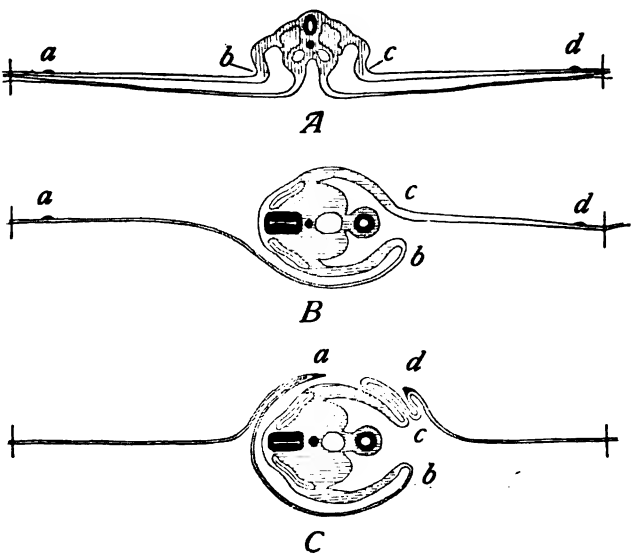

FIG. 4. *A*, *B* and *C*. Diagrams to represent the effect of rotation of the embryo on the amniogenous somatopleure. *a* represents in all figures the position of the ectamnion on the left (lower) side; *d* represents in all figures the position of the ectamnion on the right (upper) side. *b* and *c* represent the junction of amnion and body-wall on left and right sides respectively. In Fig. *A*, *a–b* and *c–d* are equal. In Fig. *B*, rotation of the embryo is assumed to have taken place without formation of the amnion; the distance *a–b* has become greater than *c–d*. In Fig. *C* is represented rotation of the embryo with synchronous formation of the amniotic folds, as is actually the case; *c–d* is inevitably thrown into secondary folds. The vertical lines at the extreme right and left represent the margins of the pellucid area.

Even though the difference may be partly compensated for by drawing of the embryo to the left, the tendency would be to stretch *a-b*. If there were no such compensation and *a* and *b* were practically fixed points, the length of *a-b* at the conclusion

of the rotation would much exceed that of *c-d* (Fig. 4, *b*); and if during this process there were actual independent growth of *a-b* and *c-d*, the latter would of necessity be thrown into folds, but not the former. Finally, if the amniotic folds were forming at the same time (as is actually the case) the right one would inevitably be thrown into secondary folds by the approximation of points *c* and *d* (Fig. 4, *C*).

Study of the fusion of the amniotic folds in actual section shows (1) that the line of fusion of the opposite amniotic limbs is over the dorsal surface of the embryo *only so long as the latter lies flat on the yolk*, and does not follow the turning of the embryo on to (usually) its left side; the consequence is that after rotation of the embryo the line of fusion lies over the upper (right) side of the embryo, often opposite the horizontal level of the intestine

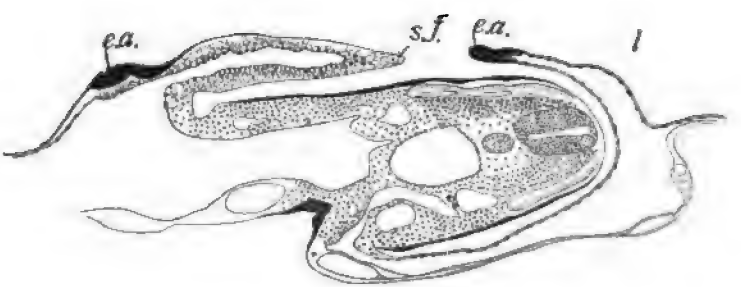

FIG. 5. Transverse section of an embryo of about 48 hours (Duval) showing the position of the ectamnion on the right and left sides. University of Chicago Embryological Collection, No. 689. *e.a.*, ectamnion; *l.*, left; *s.f*, secondary fold of amnion on the right side. The great differences in the thickness of the amnion of the right and left sides should be noted.

(Fig. 6). Thus one fold of the amnion passes all the way from the under side over the back of the embryo and around on the other side to the line of fusion, and thus is several times as long as the opposite limb. (2) Moreover, the amniotic fold of the right side is invariably thicker than that of the left side, and is always thrown into secondary folds at the place of turning (Fig. 5 and Fig. 6). These conditions are satisfactorily explained, as noted above, by the mere turning of the embryo on its side.

One must therefore distinguish in the upper limb of the amnion two kinds of folds: (1) The ordinary amniotic fold induced by the fusion of the right and left rudiments and (2) *secondary*

folds formed simply by the process of twisting of the embryo. This distinction is of importance in interpreting the results of the experiments.

Hirota (94) notices the secondary fold on the upper side and says: "It seems to owe its origin to the presence of the sero-amniotic connection. . . . It is always on the right side of the connection, and is pushed on towards the left. There takes place no folding before the allantois appears, and the longitudinal ex-

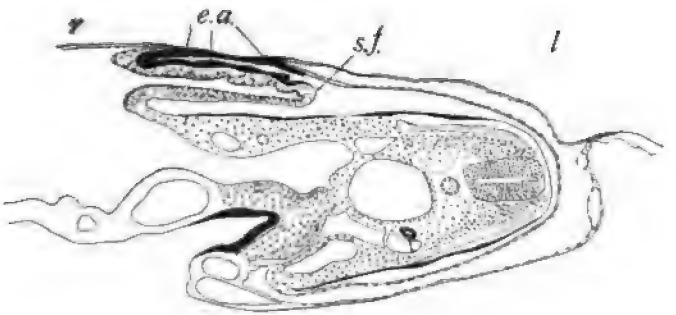

FIG. 6. Section of the same embryo as the preceding, 10 sections (150μ) in front of Fig. 5. The section passes through the place of fusion of the right and left folds. The secondary fold of the amnion is well shown on the right side. Letters as in Fig. 5.

tent of the fold depends on the extent of the sero-amniotic connection." "Its form and extent are variable." "It is not clear what significance this fold has." "At both extremities of the sero-amniotic connection the amnion is also slightly folded longitudinally."

These secondary folds of the amnion are very transitory except in two regions: (1) Above the hind end of the heart (apex of ventricle) and continuing a short distance behind it; (2) in the region immediately in front of the allantois, at 60–70 hours, thus in the neighborhood of the final closure of the amniotic folds. The former are of very constant occurrence and persist a long time (Fig. 3). The latter are relatively slight and inconstant. Hirota is thus mistaken in saying that these folds do not appear until the formation of the allantois.

The secondary folds in the neighborhood of the heart are always on the upper (right) side; they first appear at the time

of rotation of the embryo, and are coincident with the closure of the amnion (Fig. 3); they persist until the body-wall is completed behind the entire heart. They are not, in my opinion, exclusively folds of the amnion, but extensions of the body-wall for enclosure of the region of the heart and liver. The direct cause of their formation is, however, the rotation of the embryo with extreme growth of the body-wall contiguous to the amnion, and fixation of the outer end of this limb of the amnion by the amniotic suture.

Elsewhere the effect of the twisting of the embryo is rapidly compensated so that the secondary folds of the right half of the amnion do not persist long except in the region of the allantois, where slight inconstant secondary folds may continue longer.

B. *Experimental.*

1. *Experiments on the Head-fold of the Amnion.*

Experiment No. 57.

Age of the embryo at the time of operation, 33 hours[1] (Duval).

Operation.—The blastoderm was cauterized lateral to the right optic vesicle with a needle (Fig. 7) so as to make a large opening. At the time of the operation only the most anterior horse-shoe-shaped segment of the ectamnion was present (cf. Fig. 1), and this was destroyed only on the right side of the embryo. On the left side, therefore, the amniotic fold was free to form to the extent that it is independent of the opposite fold. The right optic vesicle was slightly injured, as the results of the experiments show. In opening the egg for the operation, the blastoderm was

[1] In describing the various experiments, the age of the embryo at the time of the operation will not be given as the actual number of hours in the incubator, because the variations in point of actual development after the same period of incubation are so extreme. It is not possible either to make accurate measurements of the living embryo or to determine the number of somites present, on account of the loss of time and danger of exposure of the embryo. A rough sketch of the embryo was always made at the time of the operation, and this is sufficient to identify it with the various stages figured in Duval's atlas. The age is based on this identification. Thus the given age at the time of operation in these experiments represents a certain definite stage of development. On the other hand, the length of time that elapsed from the experiment to the time of reopening the egg is always given literally.

also inadvertently torn just back of the embryo, and this opening also appears in Figs. 8 and 9. This, however, was without any noticeable effect on the subsequent development.

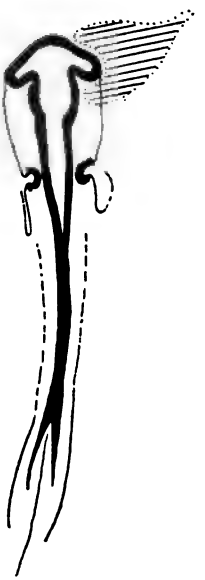

FIG. 7. Experiment 57. Operation diagram. Outline of embryo of chick of about 33 hours, after Duval. The ruled area to the right of the head indicates the area of the blastoderm destroyed by the heated needle.

Examination of the Resulting Embryo.—The egg was reopened 48 hours after the operation. The heart was beating vigorously; the hole made in the blastoderm by the operation had not closed, and a good deal of yolk had escaped through this and overlay the blastoderm. The embryo was well developed, corresponding to the stage of 70–80 hours (Duval), and apparently normal in all essential respects. (A defect in the right eye was evidently a direct result of the operation.) The head of the embryo had slipped through the hole in the blastoderm and was suspended in the yolk (Figs. 8 and 9).

The embryo was cut into 250 sections of 15 μ thickness. Around the edges of the opening made by the operation the somatopleure turns over and becomes continuous with the splanchnopleure, ectoderm with entoderm, and mesoderm with

mesoderm. In places one cannot determine where the ectoderm leaves off and the entoderm begins.

Amniotic Rudiments of the Left Side. — A short distance in front of the margin of the opening there is a sharply defined fold of the somatopleure capped by an ectodermal thickening that

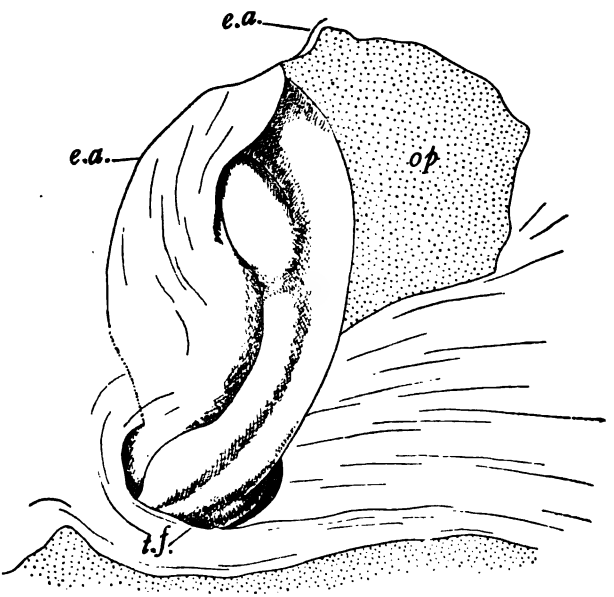

FIG. 8. Experiment 57. Upper surface of blastoderm. *op.*, aperture in the blastoderm made by the operation; *e.a.*, amniotic rudiment of the left side; *t.f.*, tail-fold of the amnion. The stippled area behind the embryo represents an aperture in the blastoderm accidentally made in opening the egg for the operation.

represents the head-fold and left lateral fold of the amnion. The extent of this fold is indicated by the line *e.a.* on Fig. 8. It begins as a sharply marked fold at the most anterior angle of the opening, and passes back, at first along the edge of the opening, later a short distance from it, to the left of the embryo. It very distinct (Fig. 10, *l.a.f.*) to the point where it is indicated as is a broken line; in this region the fold has disappeared, but the thickening of the ectoderm (ectamnion), may be traced back to the tail-fold with which it becomes continuous as indicated in the drawing (Fig. 8). At no place, until the tail-fold is reached, is the somatopleure internal to this line thrown into folds. By

reference to the figure and to the description of the operation it will be seen that the line of this fold represents the continuation of the left amniotic rudiment, which was not injured by the operation.

I conclude, therefore, that when the amniotic rudiment of one side is left free to develop after destruction of the rudiment of the other side just prior to the formation of the head fold,

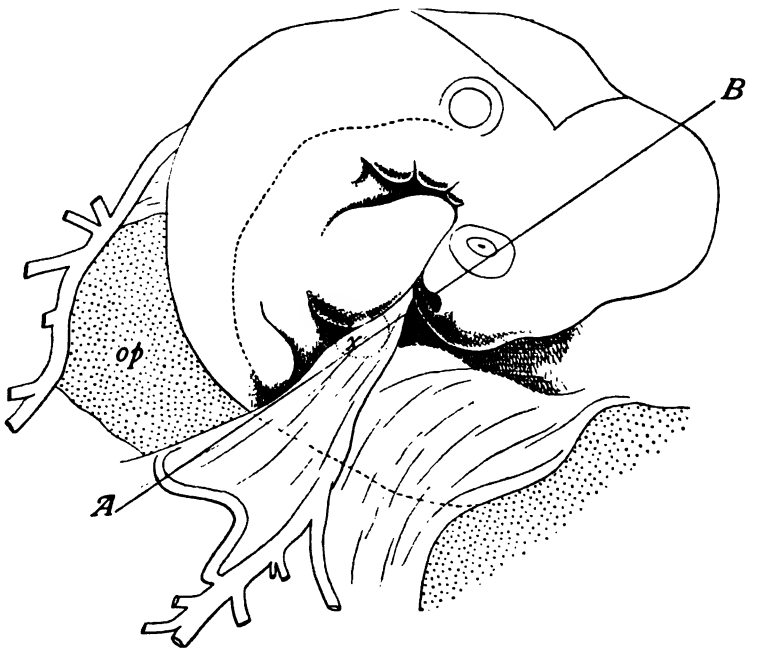

FIG. 9. Experiment 57. Under surface of the blastoderm. There is no amnion. The right eye is defective. x marks the location of the secondary amniotic fold shown in Fig. 10. A–B, plane of section shown in Fig. 10. Letters as in Fig. 8.

the ectamnion is propagated in the normal fashion and induces the formation of a low fold, but that the amniogenous somatopleure is unable to raise itself around the body of the embryo. The growth of the amniogenous somatopleure appears to be less than normal.

Amniotic Rudiments of the Right Side. — On the right side, on the other hand, a well-developed fold appears at the place where the extra-embryonic somatopleure becomes continuous with the

body-wall (section 138, Fig. 10) and extends to section 167, where it suddenly ceases, a distance of about 0.5 mm. The location is indicated by x on Fig. 9, and Fig. 10 shows it in section.

The formation of this fold is not induced by the ectamnion because the line of the latter (Fig. 10, *r.e.a.*) may be recognized some distance lateral to the fold, through it is very slightly developed. The fold in question is immediately back of the heart on the right side of the body. It is not, in my opinion, a true amniotic fold, but belongs to the category of normal secondary folds of the amniogenous somatopleure produced by the turning of the embryo, with which it agrees precisely in position and appearance. This conclusion is reinforced by the following consideration : in this embryo the roots of the vitelline veins are prolonged forward to an abnormal extent, and the right vein is fused to the somatopleure lateral to the fold (Fig. 10). As the

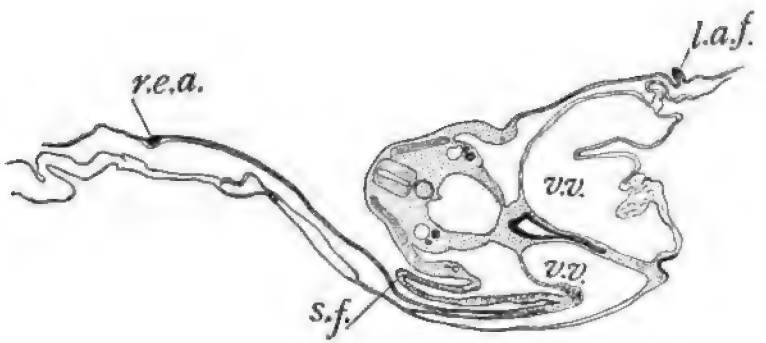

FIG. 10. Section through the embryo of experiment 57 along the line *A–B* of Fig. 9. *l.a.f.*, left amniotic rudiment; *r.e.a.*, ectamnion of the right side; *s.f.*, secondary fold of amnion on the right side; *v.v.*, vitelline veins.

embryo turns, therefore, the somatopleure between the vitelline vein and the body-wall must be folded to the extent that the turning approximates the body-wall to the vein, because the fusion prevents the somatopleure from being pushed peripherally. As already said, therefore, this is not a true amniotic fold.

The prevention of the formation of the head-fold, by destruction of the rudiment of one side, operates to prevent the normal elevation of the amniotic fold on the opposite side; and thus it is experimentally demonstrated that the coöperation of right and left folds is necessary for the normal mode and direction of

growth of the amniotic rudiments. The height of the fold on the uninjured side is a measure of the power of independent elevation of a single amniotic fold.

On the other hand the existence of the ectamnion on the right side, though in a rudimentary state, and the differences in finer structure of the somatopleure on the two sides of this line indicate that the distinction between amniogenous and choriogenous somatopleure is attained by the normal development of the somatopleure as a whole, and not simply as a result of their separation after fusion. However, the relatively rudimentary condition of the ectamnion on the injured side shows that the earlier stimulate the growth of the latter formed parts; otherwise we should expect to find the ectamnion equally developed on both sides. The ectamnion of the right side does not exactly join the tail-fold.

Tail-fold. — The tail-fold of the amnion may be well seen in Fig. 8. So far from compensating in any way for the absence of head and lateral folds, it is of even less than its normal extent, a fact indicating (possibly) that normally its growth is stimulated by the traction of the anterior section of the amnion.

Experiment No. 36.

Age of the embryo at the time of operation forty-six hours (Duval).

Operation. — The operation consisted in the insertion of a heated needle just in front of the heart (see Fig. 11). Examination of the sections of the resulting embryo shows that the injury involved the left optic cup slightly, and that the head-fold of the amnion which extends back beyond the heart at this stage, stuck to the needle and was stripped off, carrying with it a certain amount of the adjacent somatopleure. This was not observed at the time of the operation, but the conclusion is rendered positive by the subsequent examination of the embryo.

Examination of the Resulting Embryo.

The egg was reopened and the embryo preserved forty-eight hours after the operation. The embryo (Fig. 12) appeared like a normal embryo of about the ninety-sixth hour. The limb-buds were well started, and the allantois extended out beyond

the embryo, but towards the dorsal surface; the flexures were normal. The striking thing was the apparent entire absence of the amnion; the embryo lay naked on the surface of the blastoderm, to which it was attached, in the same manner as a selachian embryo by a very broad somatic and splanchnic umbilicus.

In the normal embryo of this age the amnion is completely closed, and the body-wall of the embryo has, therefore, lost all connection with the chorion.

FIG. 11. Experiment 36. Operation diagram. Outline of embryo of chick of about 46 hours, after Duval. The ruled area shows the site of the operation with the heated needle. For description of the operation see text.

This embryo was cut into 625 transverse sections. These confirm the general absence of the amnion, and at the same time furnish additional data. Back to about the 354th section (forty sections behind the heart), the somatopleure beneath the embryo is entirely missing; evidently it had been torn away by the operation and had not been replaced. Throughout this region the extra-embryonic somatopleure begins on each side of the embryo with a free edge. A short distance behind the heart, folded portions of the original amnion appear lying in the gap in the somatopleure, and continuous with the midventral line of the body-wall. Beginning with about the 371st section (see Fig. 13) the body wall is open ventrally, and is continuous with the

extra-embryonic somatopleure on one side, while on the other the original gap in the somatopleure is still open (see Fig. 13). In this region, the somatopleure for some distance external to

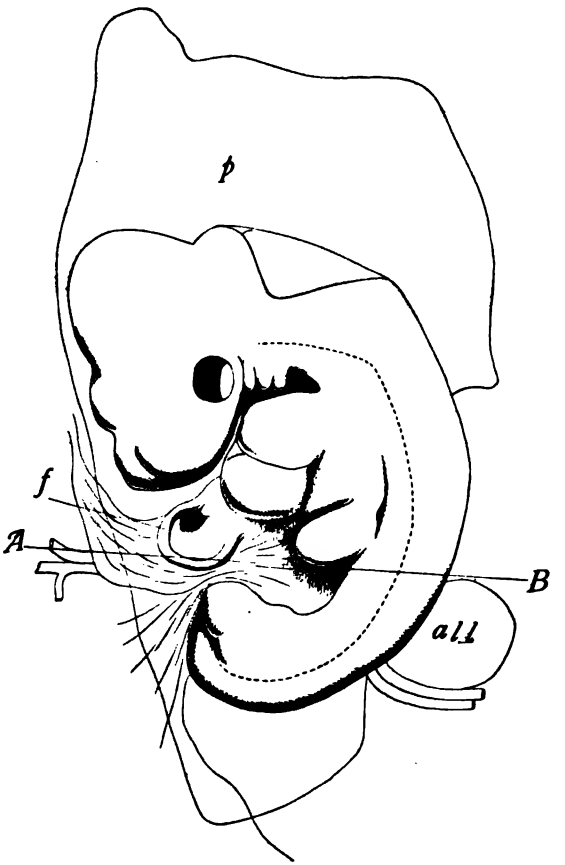

FIG. 12. Experiment 36. Surface view of embryo; upper surface of blastoderm. The embryo is anamniote, except for a rudimentary tail-fold. *all.*, allantois; *p.*, pellucid area. *A–B*, plane of section shown in Fig. 13; *f.*, fold of somatopleure.

the part destined for the body-wall is thrown on both sides into irregular folds that obviously represent the lateral amniotic folds. They rapidly decrease in size posteriorly, and *almost* completely disappear in the region extending from the 420th section back, *i. e.*, a short distance back of the fore-limbs. Beginning opposite the hind-limbs the folds again increase in size. They are very irregular and do not form the normal investment

of the tail. But beneath the latter they form a closed pocket, the usual tail-fold.

Over the entire region, extending from about the posterior edge of the fore-limb to the beginning of the hind-limb, there are no folds in the amniogenous somatopleure. This would indicate that the normal rapid growth of this region is progressively induced under normal conditions by the extension of the lateral angles of the head-fold backwards. The folds shown in the figure are only from about 354–430 and may be explained as remnants of the original head-fold, the postero-lateral prolongations of which were probably not entirely removed by the operation. These folds have not, however, united over the embryo nor have they induced formation of folds behind them. The

FIG. 13. Experiment 36. Section of embryo along the line *A–B*, Fig. 12. The irregular and incomplete amniotic folds are well shown. *e.a.*, ectamnion of the right side.

reason for this is clear when we consider that the normal process involves continuous traction on the somatopleure back of the advancing folds, for the latter are continually fusing along the dorsal line with those of the opposite side and thus are constantly, so to speak, gathering in the slack, and causing tension.

In the drawing of the entire embryo, the left side is uppermost, but at the time of the operation the right side was up. Evidently the embryo was turned over after removal of the blastoderm in the process of preparation. This explains why in the section the lower amniotic fold has the usual appearance of the upper fold. The ectamnion is visible only on the left side of the drawing; on the right side no trace of it could be found, except in the region of the tail-fold.

The results of the destruction of the head-fold of the amnion in the stage of 46 hours are: (1) Inhibition of the progressive differentiation of the amniotic zone of the somatopleure; (2) failure of the parts of the lateral folds left to unite around the embryo. The failure of the amniotic folds to unite in the region where they are best formed and are of more than sufficient length for enclosure of the embryo shows that the normal union of the folds is due to the guidance and support of the earlier formed parts of the amnion.

The tail-fold, however, forms in a fairly normal manner. The actual abnormalities in this fold are probably secondary, that is, probably due not so much to direct disturbance of the amnion itself as to the freedom of movement of the embryo permitted by the absence of the head-fold, resulting in the withdrawal of the tail of the embryo from the forming tail-fold.

The body-wall is unenclosed for 113 sections; in a normal embryo of about the same age the body-wall is unenclosed for about 55 sections. Thus it would appear that the closure has been delayed.

Experiment 60.

Age of the embryo at the time of operation about 33 hours.

Operation.—The blastoderm was cauterized just lateral to the right optic vesicle, as in experiment 57, producing a large opening (Fig. 14).

The egg was reopened 72 hours after the operation, and a large, finely developed vascular area was seen with apparently no embryo. But more careful examination revealed the naked hind quarters of an embryo sticking up near the center of the vascular area, the whole trunk and head of which were plunged through the blastoderm into the yolk. The head and trunk of the embryo had slipped through the hole made by the operation into the yolk-sac, and the edges of the blastoderm around the original opening had fused in such a way as to close around the hinder part of the embryo. A large part of the vascular area was cut out and the embryo was gently floated into a watch crystal of physiological salt solution. Turning over the blastoderm, the embryo was revealed entirely without an amnion (Fig. 15). Not even the tail-fold was found.

The embryo is represented in Fig. 15 as it lies on the reversed blastoderm, the entodermal face of which is up. The allantois is well developed and lies in a special enlargement of the body cavity behind the embryo.

FIG. 14. Experiment 60, operation diagram. Outline of embryo of chick of about 33 hours, after Duval. The ruled area indicates the region of the blastoderm destroyed by the operation.

In this experiment, as in experiment 57, only the right limb of the ectamnion of the prospective head-fold was destroyed; and the consequence of this is in both cases the suppression of the amnion with the exception of the tail-fold. In this case the various membranes have been so confused by the curious position of the embryo and by various secondary fusions that it is quite impossible to determine the behavior of the uninjured rudiment of the amnion of the left side. A single section may serve to illustrate one of the very peculiar conditions (Fig. 16). Lying above the embryo is seen the blastoderm composed of the somatopleure and splanchnopleure. The body-wall of the embryo has fused with the splanchnopleure in such a way that the two are directly continuous on both sides, and the body wall may be traced directly into the wall of the intestine. The result

of this fusion must have been an opening on each side into the yolk-sac; but this has been roofed over by extension of the

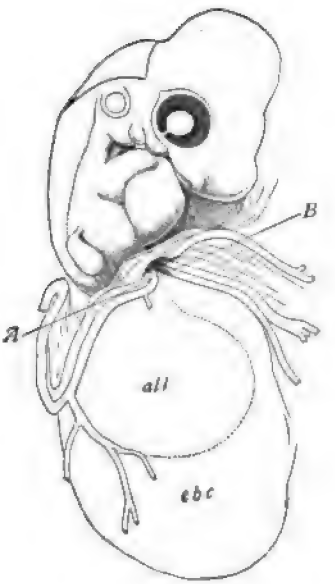

FIG. 15. Experiment 60. Under *i.e.* entodermal, surface of the blastoderm. The embryo is anamniote, but otherwise quite perfect. *all*, allantois; *e.b.c.*, extra-embryonic body-cavity; vitelline arteries and veins shown *A-B*, plane of the section shown in Fig. 16. The embryo was suspended within the yolk-sac, as described in the text.

blastoderm surrounding it. Farther back the wall of the intestine becomes continuous with the extra-embryonic splanchnopleure.

In the region of the tail rudiments of the tail-fold of the amnion are found.

Two other completely anamniotic embryos (numbers 112 and 124) were produced by experiments similar to those already described. Both of these had passed through the hole made in the blastoderm and were suspended within the yolk-sac. One of these was much farther developed than number 60. They confirm the general results of the dependence of amnion formation on the presence of the head-fold. They possess other definite lesions, the effects of which will be described in another paper.

Condition of the Allantois in Anamniotic Embryos.

The allantois is well formed in four of these embryos; one (No. 57) was too young to show it externally. It is obvious that in the absence of the amnion the growth of the allantois

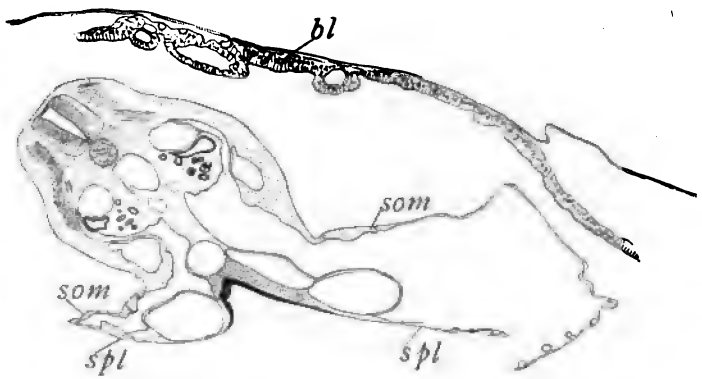

FIG. 16. Experiment 60. Section along the line *A-B*, Fig. 15. *bl.*, blastoderm overlying the embryo; *som*, somatopleure; *spl.*, splanchnopleure. On the right side there is a break in the continuity of somatopleure and splanchnopleure; this was evidently produced in the preparation, as the continuity is perfect some distance in front, and also behind.

must be attended with difficulties. When the amnion is normally formed a large free space is created above and around it, into which the allantois can freely spread. The absence of this space causes compression of the allantois, and changes the direction of its growth, but I do not think that the latter is much impeded. The mechanical force of the expansion of the allantois causes separation of the somatopleure and splanchnopleure to proceed more rapidly in its immediate vicinity than elsewhere (see Fig. 15). In experiment 124 the greater diameter of the allantois exceeds the greatest length of the embryo. I see no reason why this process might not provide all necessary space for its expansion. It might be, however, that the resistance offered would tend to cause accumulation of the products of excretion in the body of the embryo, and thus gradually poison it.

2. *Experiments on the Tail-Fold of the Amnion.*

I have also made a number of experiments on destruction of the tail-fold of the amnion. The results are in most cases com-

plicated by conditions that do not properly belong to the subject of this paper. There is but one uncomplicated case (exp. 18). In this experiment the hind-end of the embryo was cauterized immediately after the appearance of the tail-bud (Fig. 17), thus

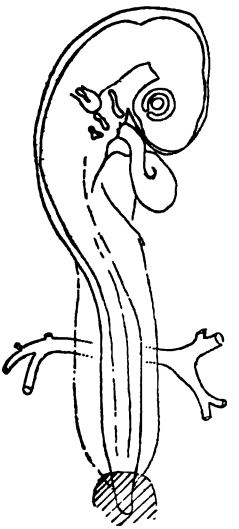

FIG. 17. Experiment 18, operation diagram. Outline of embryo of chick of about 52 hours, after Duval. The ruled area represents the part destroyed by the heated needle.

destroying the tail-fold of the amnion. When the egg was re-opened forty-eight hours later, a well-developed embryo of about five days was found in which the amnion ceased with a free edge immediately in front of the hind-limbs (Fig. 18).

The conditions of the membranes in this embryo are otherwise very complicated and difficult to understand. Thus there is in addition to the amnion a fold of the blastoderm surrounding both amnion and embryo (Fig. 18). In the posterior half of the embryo the body-wall is directly continuous with the wall of the intestine as in 60. As this embryo will come up for description elsewhere, I shall not dwell further on this topic.

The fact that stands out distinctly is that the tail-fold of the amnion has not regenerated and that the head-fold has not compensated for the absence of the tail-fold by continuing its growth backwards. However, I have a number of embryos in

which a complete amnion has been found without any tail-fold. These embryos, are, however, defective at the hind end, so that

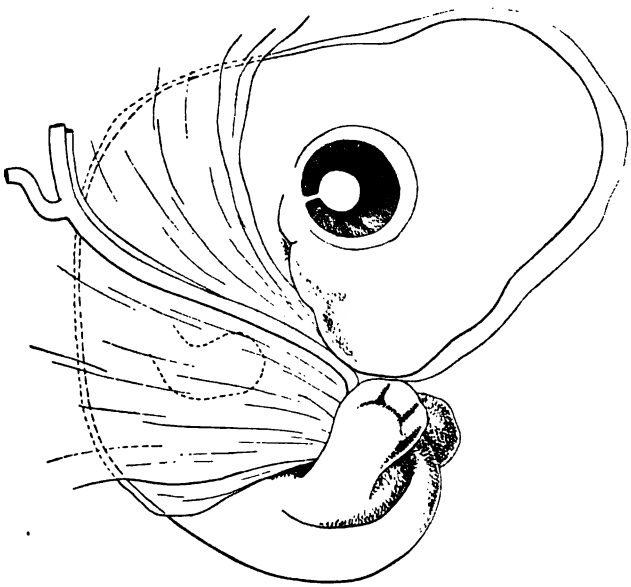

FIG. 18. Experiment 18. The embryo 48 hours after the operation. The tail-fold of the amnion has not regenerated. The amnion ends with a free edge in front of the hind-limbs. A fold of blastoderm is wrapped around the embryo and amnion. Under surface of blastoderm.

one has not to attribute any work of supererogation to the anterior lateral folds to explain the complete closure. This also will be discussed elsewhere.

GENERAL DISCUSSION.

The formation of the amnion of the chick seems to be a process with extraordinarily slight power of regulation.[1] A slight injury to part of its early rudiment sets the whole process astray. It is thus an extremely good example of correlative differentia-

[1] Barfurth ('02) notes incidentally in one of his experiments "die Amnion war regenerirt." As I understand him, he means by this simply that an aperture made in the amnion in the course of an experiment on the eye closed up. I can confirm this from my own observations. I have found that even considerable tears made in the amnion *after its formation* may close completely.

tion. The correlations in the development of the amnion are of three kinds:

1. *Mechanical.* — Under this head I class the elevation of the lateral amniotic folds, which takes place only after the establishment of the head-fold, and which is omitted, if for any reason the head-fold fails to appear or is destroyed.

2. *Trophic Stimulation.* — Under this head I class the influence of the traction exerted by the union of the right and left amniotic folds on the amniogenous somatopleure, and the influence of the turning of the embryo on the amniogenous somatopleure of the left side. The influence of the traction in either case is to increase the extent of the amniogenous somatopleure, in part (presumably) by stimulating its growth, in part undoubtedly by mere stretching. If, owing to failure of formation of the head-fold, such traction is not exerted on the somatopleure it does not expand nearly to the normal extent.

3. *Differential Stimulation.* — Under this head I class (doubtfully) the propagation of the ectamniotic thickening along the somatopleure; though this may be a process of self-differentiation.

Self-differentiation of the Formation of the Amnion. — The formation of the original rudiments of the ectamnion may be a process of self-differentiation, though the definite relation of the anterior and posterior rudiments to the head and tail respectively suggests correlation with their formation. Moreover, a slight histological differentiation appears between the amniogenous and choriogenous somatopleure, before, and even in the absence of, the formation of folds, which is apparently not correlated with any other of the processes observed.

Beyond this mere classification I do not desire to go at present, but will reserve a general discussion of principles until the completion of other parts of the present series.

In conclusion I simply summarize the results:

I. *Morphological.*

1. The amnion is primarily an organ of the ectoderm in the chick. The ectamnion first forms in front of the head and differentiates progressively backwards towards the posterior end,

where it is met by the posterior ectamnion differentiating forwards. Thus the amniotic zone of the somatopleure is marked off from the chorionic zone.

2. The head-fold is formed from the ectamnion with the coöperation of the amnio-cardiac vesicles and of the proamnion which is depressed between the former. The immediate prolongation of the head-fold is produced by the progressive fusion of the ectamniotic rudiments backwards, and it includes only an extremely small part of the proamnion.

3. The tail-fold is likewise formed primarily by the ectamnion with participation of the amnio-allantoic enlargements of the body-cavity.

4. There are certain constant secondary folds in the upper (right) limb of the amnion produced by the turning of the embryo. These persist longest in the region of the heart and immediately behind it.

II. *Experimental.*

1. Destruction of the anterior ectamniotic rudiment of one side prior to the formation of the head-fold of the amnion results (*a*) in permanent absence of the amnion back to the hind-limbs (exp. 57); (*b*) in inhibition of the growth, and almost complete suppression of the folds of the amniogenous somatopleure of the uninjured side; from which we may conclude—

2. That the growth of the amniogenous somatopleure is normally induced by the traction exerted on it by the progressive fusion of the folds, and that the uprising of the folds is due to the lifting power of the same process of fusion.

3. The tail-fold and posterior lateral folds cannot replace the anterior lateral and head-folds, nor can the latter replace the former.

4. Not only the initiation, but also the progress of the formation of the anterior lateral folds is dependent upon the perfection of the head-fold (exp. 36).

5. The absence of the amnion has, at least for a time, only a limited effect on the development of the allantois.

6. Inasmuch as the embryo may develop perfectly normally to the stage of five or six days without the amnion, it is obvious that the functional significance of the latter must be slight during

this period. It yet remains to be determined how far the embryo may develop without the amnion (see quotation from Dareste below).

7. There is a certain relation of interdependence between the formation of the amnion and the body-wall. In the absence of normal formation of the lateral folds of the amnion the closure of the somatopleure to form the body-wall proceeds more slowly than usual.

Dareste ('79) has observed total absence of the amnion in embryos of the chick. The condition was not, however, produced experimentally. His observations and conclusions are given in the following quotations:

"J'ai signalé, depuis longtemps, l'arrêt de développement de l'amnios et les anomalies nombreuses que cet arrêt partiel détermine chez l'embryon. C'est la cause la plus fréquente des monstruosités simples. Il y a des cas, beaucoup moins nombreux, il est vrai, dans lesquels l'amnios fait complétement défaut. L'embryon est alors en continuité directe, par son enveloppe cutanée, avec le feuillet séreux du blastoderme, qui ne s'est pas plissé pour former la poche amniotique. J'ai vu, dans plusieurs de ces cas, l'embryon se constituer d'une manière parfaitement normale. La paroi thoraco-abdominale s'était complétement formée, et la continuité de l'embryon avec le feuillet séreux constituait une sorte de cordon ombilical. L'allantoïde sortant de l'abdomen par se cordon s'était engagé entre le feuillet séreux et le feuillet vasculaire."

"Les embryons, ainsi privés d'amnios, peuvent vivre pendant un temps assez long. J'ai constaté l'absence compléte de l'amnios sur en embryon de treize jours, qui était plein de vie et parfaitement normal. Rien ne pouvait faire penser qu'il mourrait prochainement. Il est très-probable cependant qu'il n'aurait pas atteint l'époque de l'éclosion. L'absence de l'amnios aurait mis obstacle au développement complet de l'allantoïde: ce qui aurait produit l'asphyxie de l'embryon, comme je l'ai montré depuis longtemps. Le plus ordinairement l'absence de l'amnios amène la mort précoce de l'embryon. Souvent aussi elle détermine, dans son organisation, des modifications tératogéniques profondes."

" Toutes des observations nous font connaître le rôle physiologique de l'amnios dans la vie embryonnaire. Il est bien évident que l'amnios protége l'embryon contre toutes les actions mécaniques qui tendraient á le comprimer."

HULL ZOÖLOGICAL LABORATORY, UNIVERSITY OF CHICAGO,
April, 1903.

POSTSCRIPT.

After the foregoing paper was fully printed, my attention was called to an article by Weldon in which anamniote embryos of the fowl were described, and which I had overlooked owing to the fact that the observations were included in an article entitled " Prof. de Vries on the Origin of Species " (*Biometrika*, Vol. I., Part III., April, 1902). Partial or complete suppression of the amnion resulted from experiments to replace the water lost by evaporation in the incubator without preventing the process of evaporation itself. " A hole was made in the broad end of the egg-shell and the subjacent membranes, into which one end of a siphon, filled with water, was fitted. The other end of the siphon was placed in a reservoir of water, and the whole apparatus placed in an incubator. In from 20 to 30 per cent. of the embryos treated in this way the amnion was largely or entirely absent after incubation for three or four days."

Weldon does not discuss the mechanics of formation of the amnion, but treats the result simply as an example of a definite relation between the environment and an extremely stable character. Apparently the immediately effective factor in the experiments was the increased pressure within the shell, which, presumably, forced the embryonic area into immediate contact with the shell membrane, and thus prevented the uprising of the amniotic folds.

F. R. L.

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EXPERIMENTAL STUDIES ON THE DEVELOPMENT OF ORGANS IN THE EMBRYO OF THE FOWL (GALLUS DOMESTICUS).

FRANK R. LILLIE.

II. The Development of Defective Embryos, and the Power of Regeneration.[1]

Born has shown that young embryos of the frog possess immense power of healing smoothly cut wounds, and that the vitality of even small isolated parts is remarkable; pieces of the head or of the tail, for instance, may continue to grow and develop in spite of the complete absence of the heart, blood and blood vessels, and nervous system, for a period of about three weeks, or until the yolk contained in the cells is fully consumed. "The development of each organ progresses as far as the cut surface as well as in the normal larva, no matter what the position of the cut surface may be; the absence of the heart or the brain does not affect the subsequent processes of growth and differentiation in any marked way."

These conclusions are of great importance for the physiology of development. So far as I know, similar experiments have not been performed on the embryos of *Amniota*, and the following account may serve to fill up the gap in part. Striking differences appear between the embryo of the fowl and of the frog in regard to the vitality of defective embryos, or of parts of embryos: The chick embryo dies very rapidly after destruction of the heart, for the embryonic tissues are dependent to an extreme degree upon the circulation; it therefore follows that small parts of an embryo cannot undergo differentiation as in the frog. On the other hand, the growth of the extra-embryonic blastoderm is independent of blood supply, and may continue in eggs in which there is no embryo owing to destruction of the heart, until nearly the entire yolk is covered. This difference between the embryo of the chick and its own extra-embryonic blastoderm or the embryo of

[1] The first part appeared in Biol. Bull., 1903, Vol. V., No. 2: "Experiments on the Amnion and the Production of Anamniote Embryos."

the frog, may be explained by the simple consideration that the cells of the embryonic tissues proper in the chick are devoid of yolk or other nutriment, and hence are dependent for their subsequent growth and differentiation upon the circulation; whereas the cells of the frog embryo are loaded with food in the form of yolk; and the extra-embryonic blastoderm of the chick is a digestive organ lying on an immense reservoir of food.

The necessity of circulation for all normal development of the chick embryo beyond the stage of about 33 hours (12–14 somites) at once limits the range of defective embryos capable of development to those possessing a heart and vitelline circulation. Another limitation arises from the extreme sensitiveness of the embryo to removal of parts of the brain; although I have made over seventy experiments on the brain, none of the embryos, in which the injury extended back of the optic stalks, has developed for more than about forty-eight hours after the operation. (The operations on the head form a class in themselves and will be discussed in a separate paper.) The reason for the large number of fatalities in operations in this region is probably not due to any trophic function of the nervous system, at least in stages younger than 72 hours, but either to direct injury to the anterior end of the heart, or to malformations of the amnion consequent on the operation. On the other hand, embryos may survive the destruction of a considerable portion of the posterior end, and develop normally for several days as least. All the defective embryos to be described resulted from operations of this kind, performed, with one exception, on embryos in which the tail-bud is just forming after 50–60 hours' incubation.

A larger or smaller part of the posterior end was destroyed by cauterization. The embryos might bear complete destruction of the posterior end up to the vitelline arteries, provided these were not injured, without any apparent detriment or hindrance to the development of the uninjured parts. If the vitelline arteries were destroyed, the embryo never survived. Fig. 1 gives a view of an embryo of about the age of those used for operations. In this case 29 somites are formed; the number was certainly not over 30 at the hour of operation in any case. The somites are continued backward by the undivided segmental plate represent-

ing a considerable number of potential somites. As will be shown later, somites 26 to 32 are the ones that normally form the musculature of the leg. Thus the undivided mesoblast at this stage includes a large part of the trunk. The vitelline arteries at this time leave the embryo opposite to the twenty-first or twenty-second somites; this position appears to be very constant. Thus the theoretical limit of the experiments is from the hind end to about the twenty-second somite, because

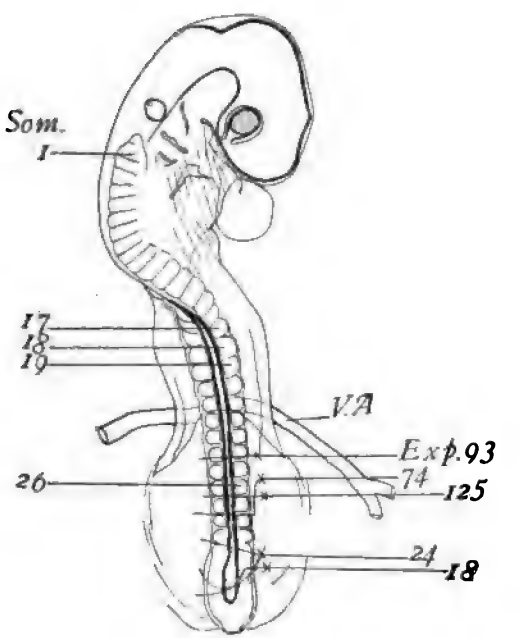

FIG. 1. Camera drawing of chick embryo with 29 somites; operation diagram. For explanation see text.

the embryo cannot live if the vitelline arteries are destroyed. The rudiment of the allantois lies beneath the unsegmented mesoblast beginning, approximately, a short distance back of the thirtieth somite.

Fig. 1 serves at the same time as a diagram of the operations. At the time of each operation a sketch was made of the embryo, and the part destroyed was indicated by shading the posterior end correspondingly. This sketch, naturally, did not show somites, so the present operation diagram is constructed from data deter-

mined after the operation. It agrees, however, fairly well with the original operation diagrams which were simply rough estimates of the amount destroyed by the operation. It should be distinctly understood that the figure is a result of study of the anatomy of the defective embryos. In further explanation it should be added, that the numbers to the right are the numbers of the experiments; those to the left of the somites. The lines leading to the numbers indicating the experiments are drawn across the body to indicate, that, in the experiment in question, all back of the line was destroyed; the cross on each line marks the junction of reference and operation lines.

A word concerning the enumeration of the somites; the somite numbered 1 possesses a short anterior process, that is probably an independent somite. It is, however, so inconspicuous in many embryos that it seemed better for the present purpose not to enumerate it. Somites 17, 18 and 19 are especially marked because they are the wing-somites, *i. e.*, the somites that will form the major portion of the bone and muscle of the wing. The twenty-sixth is the first leg somite. By this reckoning, there are three cephalic somites, or, reckoning in the incomplete one, four.

Referring to Fig. 1 again, it will be seen that the organs destroyed in such operations are: (1) The hind end of the neural tube; (2) the hind end of the notochord; (3) the mesoblastic segmental plate and often certain of the posterior mesoblastic somites; (4) the hind gut including the rudiment of the allantois; (5) the hind end of the Wolffian body and ducts.

I may say at once that no true regeneration of these structures takes place. (For discussion of this, see p. 50.) Therefore the problems of interest became narrowed down to the differentiation of the uninjured parts, and my attention has been particularly drawn to the behavior of the mesoblastic somites. In the somites we have an originally homonymous series of structures, the segmentation of which becomes strikingly heteronymous as development proceeds, some entering into the head, others the neck, others the wing, the thorax, abdomen, leg and tail. Definite somites, *i. e.*, somites in a definite numerical position in the series, form the skeleton and muscles of each of these regions. It would

be interesting to determine whether or not somites had the same rôle in defective as in normal embryos.

The numerical value of the somites in the chick seems to be normally as follows:

96-hour Chick.		Eight-day Chick.	
1, 2, 3,	Cephalic, hypoglossus region.[1]		
4 to 16,	Prebrachial, trunk.	1 to 13,	prebrachial.
17, 18, 19,	Brachial.	14, 15, 16,	brachial.
20 to 25,	Between wing and leg.	17 to 22,	between wing and leg.
26 to 32,	Leg somites.	23 to 29,	leg.
33 to 35,	Region of cloaca,	30 to 32,	region of cloaca.
36 to 42,	Caudal somites.	33 to 38,	caudal.

The count in the eight-day chick is really an enumeration of nerves. For the four-day chick the exact number of the leg somites was determined by comparison with the enumeration of nerves of the eight-day chick. The wing somites may readily be distinguished at four days, in entire mounts, by their size and by the relatively large amount of mesenchyme formed opposite them. In embryos of more than four days of age the enumeration by nerves is much the easier; and, as the brachial nerves (14, 15, 16) are very much larger than their neighbors (Figs. 3 and 5, *B.P.*), it is simplest in determining the place of a postbrachial somite or nerve to count only the somites back of the last brachial nerve. Thus the postbrachial somites 1–6 are between arm and leg; postbrachial 7–13 are leg-somites, etc.

Experiment 125.

In this experiment a very considerable part of the hind-end was destroyed at the time of appearance of the tail-bud (see operation-diagram), and the embryo was allowed to develop for about four days more (91 hours). The egg was then reopened. The vascular area covered at least three fourths of the yolk. There was no allantois. The embryo lay in large part beneath the blastoderm, but a large aperture in the latter towards the hind-end of the embryo was filled by the amnion through the transparent walls of which the embryo could be distinctly seen. A large part of the blastoderm was removed with the embryo and examination of the under surface was then made in salt solu-

[1] In this enumeration I have omitted again the incomplete anterior cephalic somite.

tion. The amnion was very well distended, and attached round the margins of the somatopleure at the hind-end. Behind this

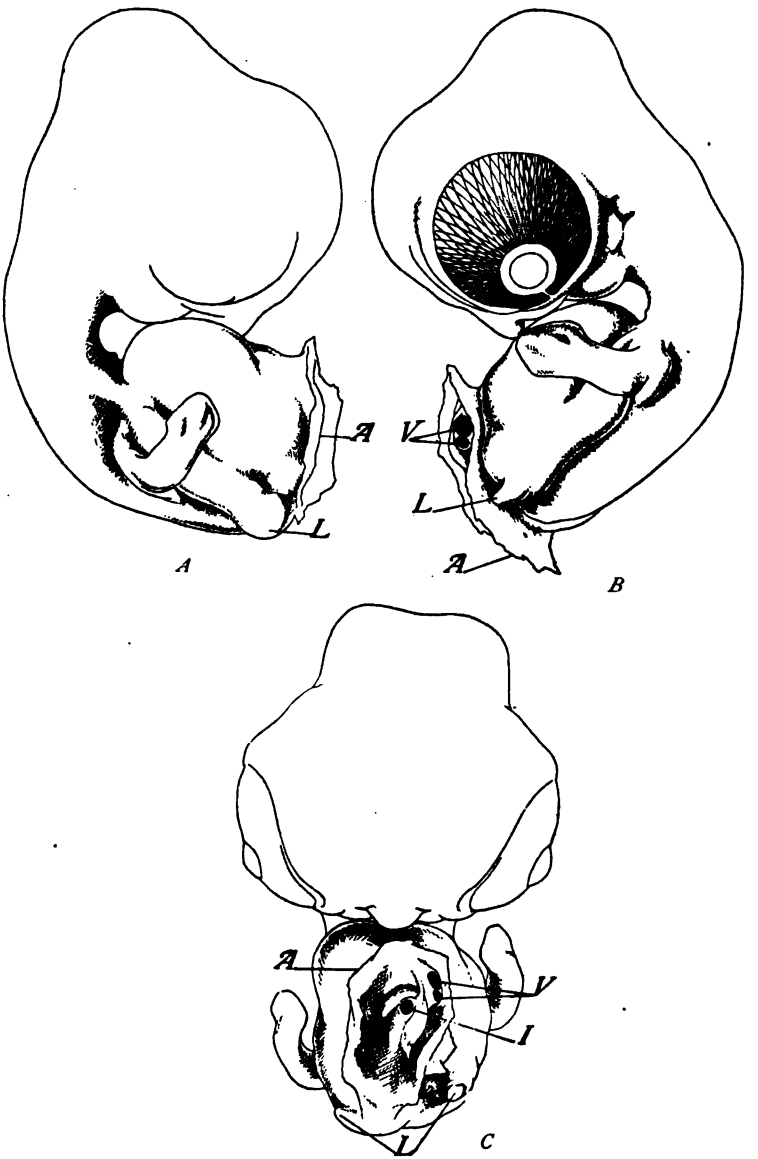

FIG. 2. Three views of a defective embryo (experiment 125) with membranes removed; total age about six days. *A*, amnion; *I*, ectopic intestine; *V*, vitelline artery and vein; *L*, rudiment of hind limbs.

the embryo was naked and there was slight ectopia of the intestine (Fig. 2, *C*).

Figures 2, *A*, *B* and *C* show this embryo from the two sides and from behind. The last figure shows especially well the attachment of the amnion and the ectopia of the intestine external to this. The hind-end of the embryo back of the vitelline vessels is wanting. On the other hand, all parts in front of this are normally developed.

On each side, at the hind-end of the Wolffian ridge, rudiments of the hind limbs are present as prominent conical stumps. From this it would appear at first sight that there has been some regeneration. However the question can be decided only by ascertaining the numerical value of the segments concerned in the formation of these stumps. The embryo was cut into sagittal sections for this purpose.

The enumeration of the post-brachial nerves is the same on both sides, seven pairs being present. The stumps are innervated only by the seventh on each side (Fig. 3). In the normal chick, the nerves innervating the leg are the seventh to the thirteenth postbrachial. Thus it would appear that only one leg somite on each side was uninjured by the operation, and this is the only one that has contributed to the formation of the rudimentary leg. None of the six somites between arm and leg has undergone any alteration of its normal numerical value. This result must therefore be interpreted in the sense of normal self-differentiation of the somites concerned.

Structure of the Leg-rudiments. — But, even though the deficiency of leg somites has not stimulated their immediate neighbors in front to any act of supererogation, it might be that the only leg somite remaining has produced more than its wont. In this connection the structure of the stumps is of interest: Owing to their positions, the left one is cut longitudinally and the right one transversely in the sagittal series. The structure is the same on the two sides (see Fig. 3); there is a single curved rod of precartilage extending out nearly to the tip of the limb; this is surrounded by a mass of dense mesenchyma, evidently premuscle tissue, and externally are the elements of the skin. Now, in the normal limb of corresponding age, the skeleton of the thigh,

crus and pes are separate chondrifications. The crus and pes are thus certainly not represented in the rudimentary hind-limb of this embryo.

In the normal chick the seventh postbrachial nerve innervates only some of the proximal muscles of the leg, those extending from the pelvis to the femur. On the principle that the muscular distribution of the nerve is confined to the derivatives of the corresponding somite, it follows that the seventh postbrachial somite contributes to the formation of only the upper segment of the leg.

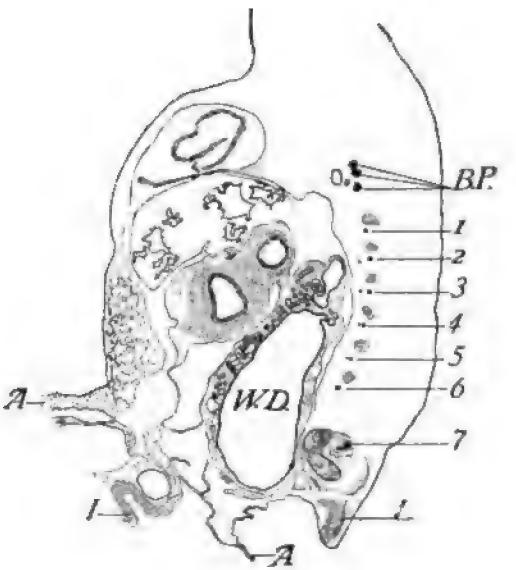

FIG. 3. Sagittal section of embryo shown in Fig. 2, cut considerably to the left of the middle line. *A*, amnion; *B.P.*, brachial plexus (nerves of wing); *I*, posterior end of intestine; *W.D.*, Wolffian duct; 1-7 first to seventh postbrachial nerves.

The absence of the crus and pes is therefore what might be expected if the principle of self-differentiation were rigidly adhered to. We have seen that, as a matter of fact, these are absent in the rudimentary hind limbs of this embryo, so we may conclude, at least, that there is no evidence that the seventh postbrachial somite has produced anything beyond the normal as a result of the absence of the following somites of the leg.

Alimentary Tract. — Practically all of the splanchnopleure of the embryo posterior to the vitelline arteries was destroyed by

the operation. In the resulting embryo, the trachea is a long tube, the lungs are budding out, the œsophagus is well formed, and the stomach is in two divisions, in the anterior of which the glands have begun to form, and the intestine is slightly coiled and opens on the hind surface, across which it is continued as a superficial strip ending in a free blind tag with a slight lumen (Figs. 2*C* and 3). The liver and pancreas are normally formed. In fact the parts present are apparently in the same condition that they would have been had the embryo been intact.

The allantois is entirely absent; and there is no evidence of any compensating growth of the intestine.

Nervous System. — The spinal cord ends bluntly, but the neural canal is prolonged backwards at its ventral angle into a short process which tapers into a bundle of neurites that may be followed a short distance, and then terminates abruptly. It would appear that the descending tracts in the cord have caused the prolongation, and have then pushed out into the adjacent tissues. A similar prolongation appears in all embryos defective at the hind-end (Fig. 3, *A*).

Excretory System. — The Wolffian ducts are much dilated, as there is no outlet for the secretion of the mesonephros (Fig. 3). No metanephric outgrowths have arisen from them, although in the normal embyro of this stage these are well developed. The metanephric region of the Wolffian ducts was of course destroyed by the operation; but, as the ducts are continuous structures, one would not anticipate that the capacity for producing metanephric outgrowths would be limited to a short stretch at the posterior end. Of course another explanation of their absence than that of limitation of potency to a short region of the Wolffian duct is possible, *i. e.*, that suitable predisposing external conditions for their formation are strictly limited (*e. g.*, that their development depends on a certain amount or kind of development of the allantois or cloaca).

Experiment 93.

In this experiment a very considerable portion of the hind-end was cauterized and removed (see operation diagram). Seventy-two hours later the egg was reopened and found living. The vascular area covered about three fifths of the yolk; the circulation was very active, and there was no allantois.

Fig. 4 is a view of the embryo in the amnion, drawn from the under side of the blastoderm. The vitelline blood vessels are attached to the center of the defective hind-end, the entire trunk back of these vessels being absent. The amnion is well distended; its line of attachment is indicated by the dotted line. Just behind this line of attachment is seen a little tag, the hind-end of the intestine. No trace of the hind-limbs is visible externally.

This embryo was cut into sagittal sections. In these there is no trace of the hind limbs, and no evidence that the posterior myotomes or sclerotomes are modified towards the formation of limbs. The embryo is not quite so far advanced in development

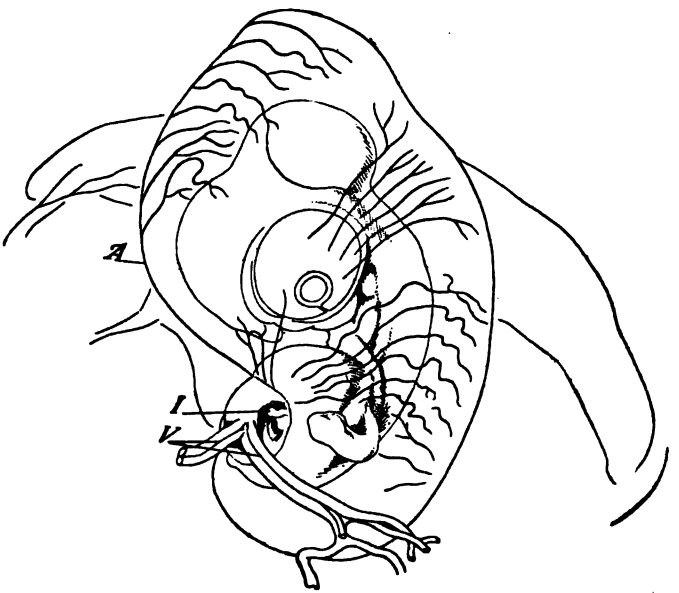

FIG. 4. Defective embryo (experiment 93) in the amnion; part of the folded under surface of the blastoderm is shown. *A*, amnion; *I*, ectopic intestine; *V*, vitelline arteries and veins.

as that of experiment 125, but it is certainly old enough to show the rudiments of the hind limbs or any part of them, were there any tendency towards their formation.

Study of the sections shows that there are only five pairs of post-brachial ganglia and nerves (Fig. 5), the fifth on both sides

being very small, and evidently partially destroyed by the operation. As the first leg-somite is the seventh post-brachial, the absence of rudiments of the hind limbs in this embryo is readily understood.

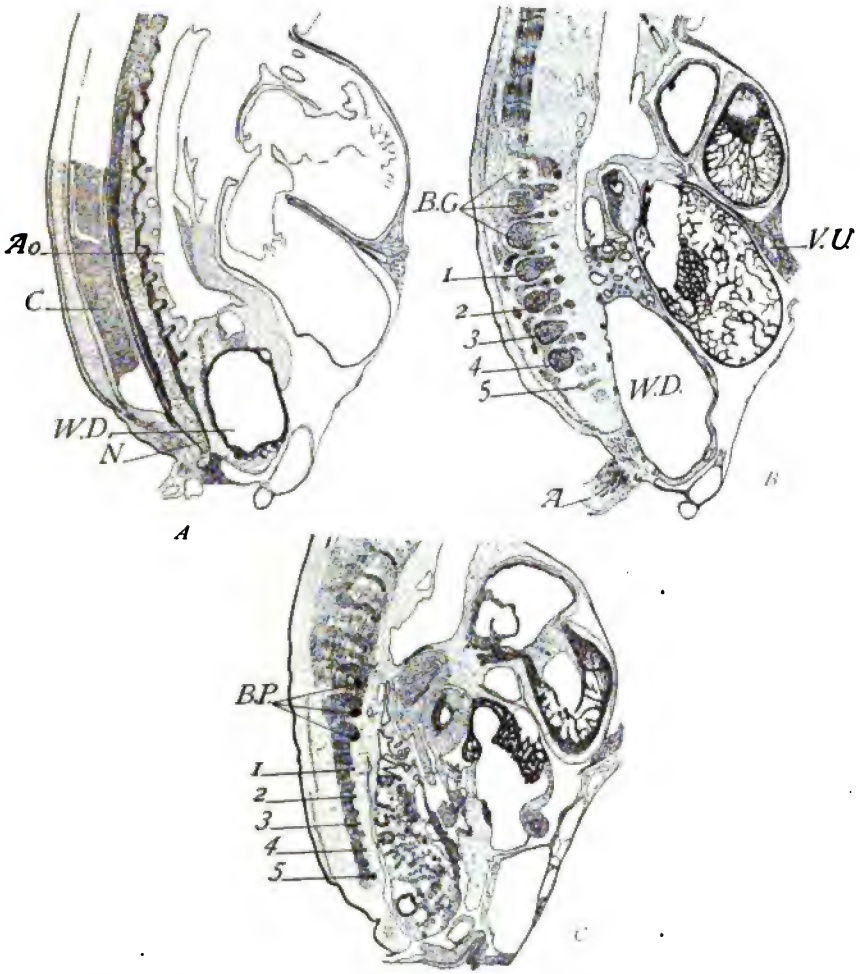

FIG. 5. Three sections from a sagittal series through the embryo shown in Fig. 4. *A*, amnion; *Ao.*, aorta; *B.G.*, ganglia of the nerves of the wing; *B.P.*, nerves of wing; *C*, cord; *N*, notochord; *V.U.*, umbilical vein; *W.D.*, Wolffian duct; 1–5, first to fifth postbrachial ganglia or nerves.

Thus there are two somites less in this embryo than in that of experiment 125. In the latter, which includes one leg somite, rudiments of the hind limbs are formed; in the present case no

such rudiments are formed, although the embryos are very much alike in every other way. It would be interesting to compare an embryo with six post-brachial somites, but I have none yet.

The central nervous system ends bluntly, except for a short prolongation of the ventral angle of the canal (Fig. 5, *A*). This condition, which is characteristic for all embryos defective at the hind-end, appears to be due to growth of fibers in the ventral motor zone, for a bunch of neurites extends back on each side of the ventral middle line nearly to the hind end of the notochord. The notochord extends backwards to the extreme posterior end, thus some distance further than the spinal cord. It thus appears, as in the other embryos of this class, to have undergone some regeneration, or at least growth, at the hind end.

The intestine opens at the posterior end, and is continued as a flat strip along the surface of the splanchnopleure. It is interesting to note that this strip has the same histological structure as the walls of the tube proper, showing that the histogenesis depends upon the character of the cells, and not upon such external factors as the form of the tube. The liver is normal. There is no trace of the allantois; but the stem of the umbilical veins may be seen, in a very rudimentary condition, running in the *septum transversum* to enter the anterior face of the liver (Fig. 5, *B*).

The *Wolffian bodies* are well developed, and the Wolffian duct much enlarged as in other anallantoic embryos (Fig. 5). Metanephric outgrowths are absent.

Summing up, we may say, that all the embryonic rudiments present have differentiated in the normal fashion. There has been no modification of the numerical value of the somites, no compensating growth, and no regeneration, unless we except the elongation of the notochord. Those embryonic organs, whose rudiment or ordinary locality was destroyed, are simply missing.

Experiment 74.

A large part of the hind end was destroyed at the stage of about 52 hours (see operation diagram). The egg was reopened about 68 hours after the operation. The vascular area covered fully half of the yolk; the heart-beat was vigorous.

Fig. 6 gives two views of the embryo. The hind end is entirely absent, and the opening is filled by the enlarged Wolffian ducts. The band of tissue between the two ducts is the hind end of the alimentary tract. The right side is seen to be less developed than the left. In the view from the opposite side, it can be seen that there is a rudiment of the hind-limb on the left side; but apparently none on the right.

This embryo was cut into transverse sections. Study of these shows that there are eight postbrachial ganglia on each side, though the eighth on the right side is smaller than on the left.

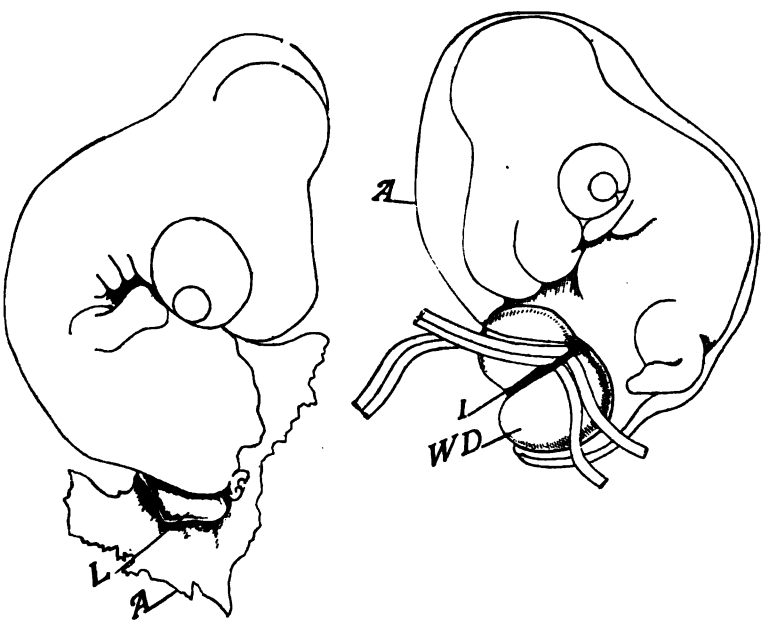

FIG. 6. Two views of a defective embryo (experiment 74). *A*, amnion; *I*, strip of intestinal epithelium; *L*, leg; *W.D.*, Wolffian duct. The vitelline blood vessels are somewhat abnormal.

On the left side the seventh and eighth postbrachial nerves enter the rudiment of the hind limb. On the right side there is no such rudiment, and the seventh and eighth nerves, which are quite large, end bluntly in the mesenchyme, almost as though cut off. The question arises why there is no limb rudiment on the right side? One can explain this by assuming that the operation destroyed the seventh and eighth postbrachial meso-

blastic somites on this side, but not the ganglia and cord of these somites (see Fig. 1). The form of the embryo indicates that this is true in part; but there is a small myotome in the eighth postbrachial somite of this side, and in the seventh the myotome is quite as well developed as on the other side. It would appear probable, then, that the most lateral portions of the seventh and eighth postbrachial mesoblastic somites were destroyed, together with the somatopleure lateral to this; either the rudiments of the hind limb were included in the destroyed parts, or their development was prevented by the scar-tissue. It is impossible to say whether or not the two somites concerned in the formation of the left leg have formed more than the normal.

The central nervous system terminates abruptly, except that the ventral portion of the canal is continued back as a narrow epithelial tube. The notochord extends some sections back of the termination of the neural tube, showing that it has been added to at its hind end since the operation.

The Wolffian ducts are immensely enlarged (see figures). There is no metanephric outgrowth. The allantois is entirely absent. The alimentary canal is normal back to the defective region and terminates in a band lying between the two Wolffian ducts (Fig. 6).

Experiment 18.

The tail-bud of the embryo was just formed at the time of the operation, and it was completely destroyed with a heated needle (see operation diagram). The injury did not extend so far forward as in the other cases mentioned and was asymmetrical (Fig. 1).

Fifty hours later the egg was reopened, and the embryo was found living, the heart beating actively; the vascular area covered about one third of the yolk.

Fig. 7 shows the embryo as it appeared from the under surface of the blastoderm. It will be observed that the amnion ceases with a free edge immediately in front of the hind-limbs; thus the tail-fold of the amnion destroyed by the operation has not regenerated, nor is the allantois visible externally, as it always is in normal embryos of this age. The hind-limbs appear normal; part of the tail at least is present.

The enumeration of the postbrachial nerves in the sections gives the following results: 1 to 6 on each side are in front of the leg-rudiments; the following seven nerves on each side enter the leg-rudiments. On the right side there is only one incomplete ganglion back of the last leg-nerve, *i. e.*, the fourteenth postbrachial; the nerve from this cannot be traced. On the left side there are four complete ganglia and myotomes back of the last leg nerve (14 to 17 postbrachial). Thus, as might be expected, the hind limbs are normal, and the cloacal region is strongly affected.

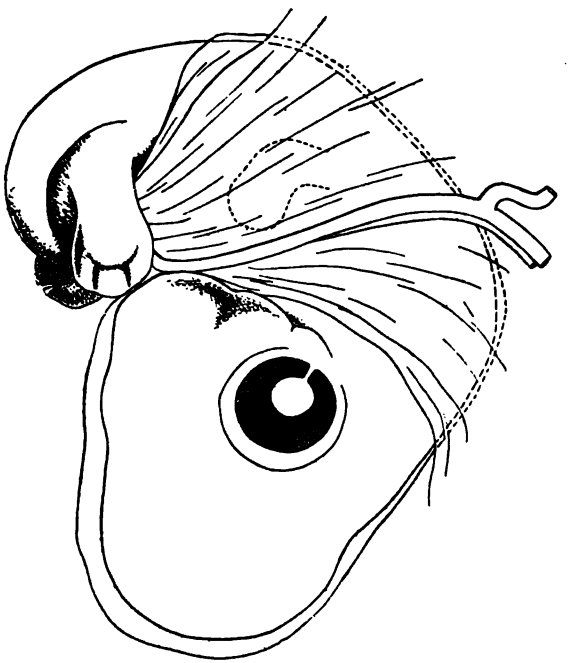

FIG. 7. Defective embryo (Experiment 18).

In the embryo of this experiment the Wolffian ducts have normal relations, but the allantois is thick-walled and undilated; the entodermal cavity of the latter opens out into a groove in the splanchnopleure, leading back to the hind-gut, which is not closed as it normally is; in fact in this embryo the cloaca is the only closed region of the hind-gut; immediately in front of the cloaca it is open ventrally. The stalk of the allantois therefore appears first as a groove on the left side; followed forwards, this

deepens gradually into a canal that penetrates into a mass of mesoblast connected with the somatopleure, and excavates a large irregular cavity in this. The mass extends forwards to the tip of the ventricle and ends in a bifurcated extremity. This represents the allantois, the main mass of which is composed of loose, very vascular mesenchyme forming an appendage to the somatopleure on the left side. On the right side the mesoblast of the somatopleure in the allantoic region is much hypertrophied and forms a large free lobe without any entodermal contents.

It is remarkable that the allantoic rudiments should show such considerable power of growth in the absence of the usual stimulus of internal pressure, which is precluded by the open groove-like connection with the intestine.

Experiment 24.

The hind end of an embryo was cauterized with the aim of destroying the incipient tail-bud and the region of the hind-

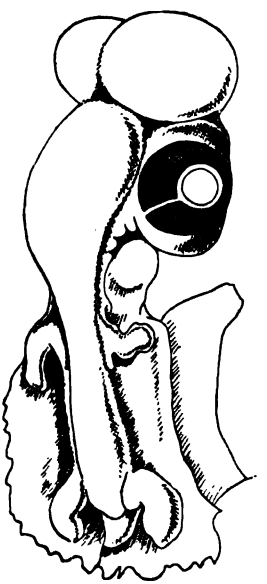

FIG. 8. Defective embryo (Experiment 24).

limbs. The egg was reopened 48 hours after the operation. The vascular area covered about two fifths of the yolk, and appeared to be entirely normal.

Fig. 8 represents the resulting embryo from the dorsal surface; the rudiments of the hind-limbs are apparently as well developed as those of the anterior limbs, and the posterior portion of the trunk is separated from the anterior by a sharp depression. The tail turns to the left, and tapers to an end beneath the left hind-limb; part of its course is hidden by a fold of the somatopleure, as shown in the figure. The depression evidently represents the anterior limit of the injury.

This embryo was sectioned. On examination it was found that, although the region behind the depression has the usual structure of the postanal region of the body, its neural tube has no connection with that of the trunk, and the notochord is absent in the anterior third. Mesoblastic somites occur in this region, but the main part of its substance is made up of loose vascular mesenchyme.

It is plain, then, that this part has not regenerated in the sense that it has grown out from the embryo; and there remains only the assumption that its parts have formed from embryonic tissue remaining in this region after the cauterization. The absence of the notochord throughout almost all of the tail shows that the destruction due to cauterization was very extensive; its absence beneath the neural tube for a considerable distance shows, that the neural tube has formed secondarily in this region; because any operation, that would destroy the notochord, would necessarily destroy the neural tube. Farther, the relation of the Wolffian ducts and allantois to the cloaca are abnormal, and the hinder portion of the intestine has no connection with the anterior portion; all of which shows that there was originally complete destruction of tissues through to the entoderm in the anterior part of the cauterized area. There seems to have been in this case a reorganization of the embryonic tissue of the caudal region.

On the left side I find ten postbrachial ganglia back to the region of the defect. Thus at least four segments of the normal hind limb-region are included, viz., 7, 8, 9, 10. The last is small, representing only a fraction of the normal ganglion. On the right side there are eleven full-sized postbrachial ganglia. Thus there are at least one and one half more metameres on the right than on the left side, and this tallies with larger size of the right hind limb and with the operation diagram.

The only interpretation of this result is that the needle destroyed a transverse section of tissue and separated the tail-rudiment from the rest of the embryo, at the same time causing some injury to the tissue of the tail; secondary union then took place. The tissue along the line of junction is peculiar in many respects, including much material, probably remnants of cauterized parts, that takes the plasma stain.

Experiment 187.

In this experiment the operation was made at a stage of about 24 hours; the medullary plate was just formed and the primitive streak was yet long; probably 3 to 5 somites were present (Fig. 9). Three spots were marked with an electrode, one behind the other in the middle line towards the posterior end of the primitive streak. The three spots were at first separate, but later examination showed them running together; so that all of the

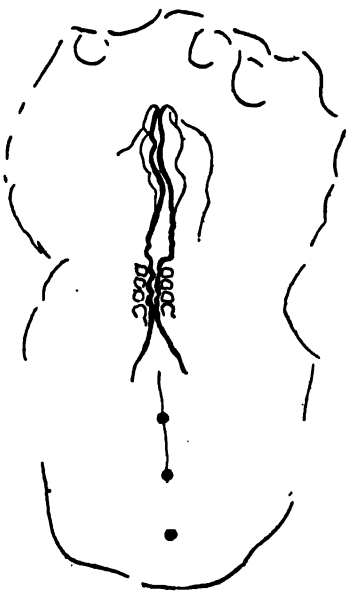

FIG. 9. Operation diagram for experiment 187; drawn from a blastoderm of the same lot of eggs used in this experiment; preserved at the time of the operation.

axial structures were destroyed in this region. The embryo was allowed to develop two days longer and was then preserved.

It will be seen (Fig. 10) that this operation has taken us in front of the vitelline arteries; having been performed before the development of the arteries, it was possible for the rudiment of the vascular system to readjust itself to the new conditions, and establish a vitelline circulation. The vitelline arteries are, however, in about their normal position, *i. e.*, about opposite the 20–22 somites, hence behind the embryo in this case. It is difficult to see why they should have this position and not be shifted farther forward, if the view of His, that the main blood vessels develop from the paths of least resistance in the primitive vascular network, be true.

On the right side of this embryo there are 14 spinal ganglia, and on the left 14 complete and part of the fifteenth; but as, in the fowl, the first two cervical nerves lack ganglia, there are present 16 spinal nerves on the right side, and 17 on the left.

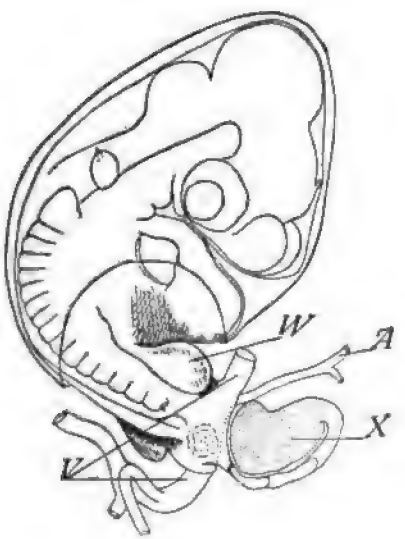

FIG. 10. Defective embryo (experiment 187). The drawing was made from the stained and cleared specimen. *A*, vitelline artery; *V*, vitelline veins; *W*, wing-bud; *X*, sac filled with blood, behind the embryo in the prolongation of its axis; tish has no connection with the vessels of the embryo.

The normal innervation of the wing is from the fourteenth to the sixteenth spinal nerves. It would thus appear, on the evidence of the nerves, that the operation has destroyed everything back of the wing somites. The wing-buds have a development that appears perfectly normal for this stage.

On the right side there are nineteen myotomes, and on the left twenty and a half. Fig. 10 shows the myotomes of the right side as they appeared in an entire mount of the stained embryo in oil; only 16 can be counted. But sections show, that the large anterior one is really three, and the nineteenth is represented by the mesoblast behind the last complete somite. If we reckon the three anterior somites as cephalic, this leaves sixteen trunk myotomes, and the enumeration agrees with that of the nerves. On the other side of the embryo there is distinctly one more cephalic myotome than on the right side; if, then, we reckon four as the number of cephalic myotomes on this side,[1] there are sixteen and a half trunk myotomes, a result that agrees with the enumeration of the nerves.

The organs in this embryo, as in the others described, have developed normally as far as the cut surface. The Wolffian ducts are enlarged by internal pressure. There has been no compensatory growth and no regeneration, if we except the notochord.

General Discussion.

The chief value of these experiments consists in the fact that they clear the ground for farther researches of a more definite character. It is probable that the results obtained will hold in general for the Amniota, and they may, therefore, be of value in the interpretation of teratological phenomena. Results that I have obtained from destruction of large portions of the brain in young embryos have convinced me that great caution must be exercised in interpreting conditions associated with anencephaly. It should be possible, and I am convinced that it is possible, to produce these various conditions experimentally. Until this is done it seems to me that conclusions, based on teratological material, as to the trophic value of the embryonic nervous system are debatable.

The following questions definitely raised by the experiments just described may be answered here in part:

1. *As to Regeneration.* — The only organ showing evidence of regeneration is the notochord. In all of the embryos described,

[1] Froriep recognizes four cephalic myotomes in the chick; the most anterior, however, becomes rudimentary at a very early stage, and soon disappears; this condition seems to have been attained on one side in this embryo, but not on the other.

the notochord extends a short distance back of the neural tube and its end is very protoplasmic; its further extension in each case is definitely limited by the surface of operation, against which it is pressed (Fig. 5, *A*). One receives the impression that its growth has been stopped by the mechanical hindrance.

One does not expect a vertebrate to show axial regeneration of the trunk, but in certain vertebrates the tail may regenerate. What is the condition in the chick embryo? No. 18 is the only case cited in which any of the caudal somites were left uninjured. In this case there was no regeneration, so far as could be judged.

The very first experiments that I performed on chick embryos were made to determine whether or not the limb-buds might regenerate. The operations were limited to the wing-buds. I found that it was possible to amputate the right one close to the body at a time when its width was equal to or greater than its length, and even later (four or five days); the wound in the amnion might close, and the amputated part remained in the amniotic cavity as evidence of the operation. In the only cases in which the embryo lived for any considerable period after the operation, there was absolutely no sign of regeneration, though the wing-bud of the opposite side increased in bulk several times.

So far as I know, the only evidence, that the organs of the chick embryo possess any different power of regeneration from those of the adult, consists of the above observations on the notochord (tissue regeneration) and of Barfurth and Dragendorff's[1] observation on the regeneration of the lens of the eye from the edge of the optic cup. The last depends on observations on a single case, and in this case the extent of injury to the eye was doubtful. Until the result is confirmed, I believe we are justified in passing it over.

There would remain, then, the general conclusion, subject only to the qualifications already noted, that the embryo of the chick possesses no greater power of regeneration than the adult.

2. *As to the Somites.* — Somites always retain their normal numerical value. But the experiments were not adapted to analyze very accurately the numerous problems presented.

[1] Dietrich Barfurth and O. Dragendorff, "Versuche über Regeneration des Auges und der Linse beim Hühnerembryo," Anat. Anz., Ergänzungsheft zum XXI. Bd., 1902.

Thus the operation destroyed in each case not only definite somites, but also all posterior to the first one injured, and the body wall lateral to the somites. With more refined methods, it may be possible to eliminate single somites from within the series, and to avoid injury to adjacent tissues. Such a technique would enable one to analyze many of the problems offered by the somites: to determine, for instance, the exact part played by them in development of the limbs, the order of origin of the most anterior somites, etc. Such problems are now being studied with partial success, and the results will be published later.

UNIVERSITY OF CHICAGO,
March, 1904.

OBSERVATIONS AND EXPERIMENTS CONCERNING THE ELEMENTARY PHENOMENA OF EMBRYONIC DEVELOPMENT IN CHÆTOPTERUS[1]

BY

FRANK R. LILLIE

WITH ONE PLATE AND SEVENTY-EIGHT FIGURES IN THE TEXT

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[1] The experimental parts of this study and the observations dealing with the living egg were carried on at the Marine Biological Laboratory of Woods Holl, Mass; the study of the preparations in the Zoölogical Laboratory of the University of Chicago.

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I. INTRODUCTION

This paper is based on the study of the egg of a single species of annelid, Chætopterus pergamentaceus Cuvier, and is a contribution to the study of the elementary phenomena of development. I have been able to extend my earlier observations on differentiation without cleavage (Lillie, '02) and to demonstrate homology of regions and cell-constituents between unsegmented eggs that have undergone differentiation and larvæ normally formed. It has been found in the course of the later study that the homology is dependent upon a very exact localization of germinal areas and specific substances in the unsegmented egg, which play essentially the same rôle whether they be divided by cell-walls or not. The microscopic composition of these substances and the relations of nuclear activity to differentiation have also been studied, and it has been necessary to include the ovogenesis, maturation, fertilization and cell-lineage in order to control the other data.

In the present paper the attempt will be made to present the substance of the entire study without too much detail, leaving the elaboration of certain parts for a later contribution. However, I shall make no apology for entering into details, because there is no other explanation of heredity than a complete account of development, and one cannot describe even a small part of so complex a thing without many words, unless one knows in advance what is essential and what is not.

The numerous cytological studies of germ-cells of the last few years have focused attention especially on the chromosome-complex, and the remarkably uniform results have established a series of propositions on so firm a basis that they seem destined

to form the working hypothesis of investigations in heredity and development for a considerable period of time. These are: (1) The constancy in number of the chromosomes in any species. (2) The persistence of a descendent of each chromosome in every cell throughout the series of cell-generations. (3) The composition of the chromosome-complex of the first cleavage-spindle of corresponding maternal and paternal groups, equal in number and in all other respects, save individual variation. (4) The biparental character of the embryonic nuclei, similar to that of the first cleavage nucleus. (5) That the individual chromosomes of both the maternal and paternal chromosome-complexes are probably qualitatively different. (6) That pseudo-reduction (synapsis) consists in union of corresponding maternal and paternal chromosomes. (7) That reduction in the number of chromosomes is effected by separation of the units of such bivalent chromosomes.

Though all of these propositions are not accepted by all cytologists, yet in the main they have become the current opinions. Enough, certainly, has been definitely established to put the hypothesis, that the chromosomes constitute the bearers of the hereditary qualities, on a definite basis. *The embryologist, therefore, has his problem defined, and it is no less an undertaking than to derive the entire body of the individual from the chromosome-complex as its germ. This is the embryological program.* It has been recognized as such in the more important recent theories of development; but hypothesis has far outstripped observation, and it is remarkable how very few observations have been recorded establishing definite morphogenic relations between nucleus and cytoplasm. Yet, if the hypothesis be true, such relations must be of the very essence of embryonic development.

The observations recorded in this paper have led me to some very definite opinions as to the relations of nucleus and cytoplasm in embryonic development. I am well aware that they cover only a very small part of this embryological program; but I venture to express the hope in putting them forward that others may be led to test and to extend them.

Our advance along these lines is very dependent on methods. The results of this study could not have been attained without the

methods employed. It is probable that centrifuging and staining intra vitam will prove to be very generally applicable, and I believe they will yield valuable results. The method of analyzing the developmental processes by suppressing the cleavage without prejudice to other morphogenic activities, has been the most valuable one on the whole. I believe that it is capable of quite a wide application even in cases where the differentiation does not proceed to the formation of cilia. Bastian ('04) seems to have witnessed such a process in rotifers, though, by an incomparable blunder, he interpreted the unsegmented ciliated ova as a definite genus of ciliate infusorian. It applies in other annelids than Chætopterus as Scott's ('06) and Treadwell's ('02) papers show, and I think there is every reason to believe that it will prove available in animals of different phyla.

II. MICROSCOPIC COMPOSITION OF THE PROTOPLASM OF THE EGG

1. The Living Protoplasm

The egg of Chætopterus is a spheroidal mass of a semifluid, transparent and homogeneous substance with a large number of granules of various sizes and optical properties suspended in it; more fluid droplets occur here and there. The living protoplasm shows no evidence of a filar, reticular or alveolar structure, except that granules or droplets may be so closely set in certain places as to produce the appearance of an emulsion. This, however, is a secondary character. The primary optical properties of the protoplasm are those of a transparent colloidal solution, the particles of which are ultramicroscopic; this may assume the most various configurations according to the number, size and arrangement of the suspended, microscopically visible, granules.

The ground substance is a suitable name for the fluid that contains and suspends all the granules and droplets; if all these were imagined removed it would preserve a faithful semblance of the egg. Thus it is regarded as forming the external pellicle and as continuous through the nuclear membrane with the nucleoplasm. Its optical properties are the same everywhere like a drop of water, but, unlike such a drop, it may exhibit different physical and chemical properties in different parts.

The Granules.—The varying microscopic character of regions of the egg is thus due entirely to the granules and droplets suspended in the ground substance. Droplets of a homogeneous transparent liquid, more fluid than the ground substance, are especially characteristic of the germinal vesicle and of the protoplasm in the neighborhood of the vegetative pole (Fig. 1). I have not, however, been able to follow their behavior very fully. The granules on the other hand are more solid bodies, though it is probable that some are semifluid; certainly all have considerable density.

By subjecting the living egg to considerable pressure one can examine it with the highest powers; my observations were made with a Zeiss 2 mm. homogeneous oil-immersion objective, apert. 130, and compensating oculars 6, 8 and 12. Under such magnifications one sees that the protoplasm of any part consists of the ground substance with granules of two kinds, viz: slightly or non-refringent granules of relatively large size, which I shall designate spherules, and very minute (less than 1μ in diameter) highly refringent granules of approximately uniform size, the microsomes. The spherules vary in size from about 2.25μ to about the size of a microsome.

In the living protoplasm the microsomes and the smaller spherules are in a constant state of tremulous agitation vibrating in rapid rhythm with considerable amplitude. The range of these movements is considerably increased if the egg is actually crushed so that the protoplasm is broken into small fragments. No one who has studied these movements, as I have done for hours at a time, could believe that the microsomes are nodal points of a network, or are connected by filaments as they appear to be in the best stained sections. One is forced to conclude that they have freedom of movement in all directions, *i. e.*, that they are suspended in a fluid medium which has no filar, reticular or alveolar structure.

The description so far applies to the substance of the germinal vesicle as aptly as to the cytoplasm; though the spherules of the germinal vesicle are probably small droplets in the sense used above; they certainly differ in many respects from the spherules of the cytoplasm. But the substance of the germinal vesicle con-

tains as large a proportion of microsomes as does the cytoplasm, and these cannot be distinguished apart in the living condition, though they can be differentiated by fixation and staining, as described below. The germinal vesicle is of course characterized also by the nucleolus described in Part III; the chromosomes were not distinguished in the living germinal vesicle. There are also large spaces filled with fluid (Fig. 1).

Microsomes may occur separately or in conjunction with spherules; in the latter case a certain number appear to be inseparably united to the spherules; no matter how much the protoplasm is broken up, the union cannot be dissolved. I am not sure that this is characteristic of all the various kinds of spherules, but it certainly is of those of the ectoplasm. There are different kinds of spherules characteristic of different regions of the egg and related to different kinds of differentiation. Their differences, arrangement and properties are considered later.

The relation between microsome and spherule is more or less problematical. From the fact that spherules may grade down in size to approximately the dimensions of microsomes, one is tempted to assume that the spherules are produced by growth of microsomes, or by their agglutination and fusion, and the same conclusion follows from other facts considered beyond. The microsomes would thus be the primitive source of these larger granules; the spherules a farther step in the processes of differentiation.

According to this view the microsomes are the primitive formed elements of the cytoplasm. The source of the microsomes themselves becomes therefore a vitally important question. There are three possibilities: (*a*) That the microsomes arise from smaller ultramicroscopic particles in the ground substance; (*b*) that they are an original cytoplasmic element multiplying by fission; (*c*) that their primitive source is the nucleus, in which event they are to be regarded as chromatin derivatives, for all the formed elements of the nucleus are chromatin derivatives, or, at least, have their base in the chromosomes.

I have observed no phenomena that indicate either of the first two possibilities, but have on the other hand repeatedly observed the transformation of nuclear microsomes into cytoplasmic micro-

somes in sufficient quantity to supply all the microsomes found in the egg. *In addition to these direct observations several important considerations unite in support of the hypothesis, that microsomes are chromatin particles.*

The direct observations concerning the structure and fate of the residual substance of the germinal vesicle (pp. 172–178) and concerning chromatin distribution in uninucleated unsegmented eggs that undergo differentiation (pp. 230–232) conclusively prove the derivation of a large proportion of the microsomes from the nucleus. Now each daughter nucleus is built up from a particular chromosome-complex, and it is a commonplace cytological generalization that chromosomes arise by the union of chromatin particles (chromomeres) of approximately the size of cytomicrosomes; this is readily observed in the egg of Chætopterus. When the nucleus comes to rest the chromosomes resolve themselves again into these ultimate formed elements of the chromatin, after passing through specific form changes. Thus the chromosomes are composed of particles of about the same size as the cytomicrosomes. The staining reaction of the chromomere and cytomicrosome is the same, in certain stages at least; newly formed microsomes take the basic stain with nearly the same intensity as chromatin particles. In the ovogenesis of Chætopterus the main source of the microsomes is the structure described by Mead ('98, pp. 193, 194) as the paranucleus as follows: "Up to the time when the egg has attained about two-thirds its full size, only a part of the protoplasm presents the loose reticular appearance; the rest remains as large purple masses, which I consider to be equivalent to the *Nebenkerne* or paranuclei of various authors (Fig. 1, *c*, *d*, *f*). These masses are not homogeneous, but resolve themselves into a cytoplasmic network, of which the meshes are much compressed, and the strands usually parallel with the surface of the nucleus, though at the periphery of the masses they fray out and become continuous with the open network which contains the yolk." I would add that the paranucleus forms a kind of cap to the germinal vesicle, from which it probably originates. The reticulum, in my opinion, is an artefact, and the essential thing about the paranucleus is that it is composed of a dense aggregation of micro-

somes, that subsequently become distributed to all parts of the cytoplasm.

These various considerations have convinced me that the larger proportion of the microsomes are derived from the nucleus. It seems improbable that there should be any *radical* difference in the origin of any, for the morphological characters of the cytomicrosomes, such as size, density, color and refringibility in life, and staining reactions, are remarkably constant. I will not deny that they may multiply outside of the nucleus; but, except in the case of the centrosome, I know of no evidence in favor of such multiplication of bodies resembling microsomes. On the other hand it is certain that the chromomeres multiply within the nucleus. If the microsomes are *extra*nuclear chromomeres, as here suggested, the doctrine of the individuality of chromosomes must be understood, as Häcker has pointed out, not in the sense that the entire substance of a chromosome is handed on through successive generations of cells, but only in the sense of persistence of part of the substance in each cell-generation. The ultimate consequences of this hypothesis of the microsomes are far-reaching, and need not be considered here.

2. *Comparison of Living, and of the Fixed and Stained Protoplasm*

In general it may be said that the sections give an extraordinarily faithful picture of the protoplasm, with one exception, viz: the appearance of a reticulum in the ground substance (Fig. 1). Practically all methods of fixing and staining employed show this reticulum. I have already mentioned one reason for supposing it to be an artefact, viz: the rapid "Brownian" movements of the very particles (microsomes) that appear in the preparations to be in the nodes of the reticulum. A second reason for this belief is the fact that the spherules may be driven through the cytoplasm in any direction by centrifugal force without destroying its capacity for development (Part III, 1, *c*). A third argument is found in the formation of pseudopodia at certain stages in the experiments described in Part IV, of such exceeding tenuity that

an alveolar or reticular structure of the pseudopodial protoplasm in any way resembling that of the sections is impossible; but such delicate strands of protoplasm are living and exhibit characteristic movements, though they grade off into invisibility. When one adds that no reticulum can be seen by any of the usual devices in the living protoplasm, and that a similar reticulum has been demonstrated to be formed in colloid solutions known to be homogeneous by the usual methods of coagulation employed in the preservation of protoplasm, it certainly seems much more reasonable to conclude that the reticulum is an artefact in the preparations.

The preparations, however, in other respects greatly extend the observations that it is possible to make on the living protoplasm. Thus they enable one to distinguish different classes of microsomes and spherules by microchemical reaction, to differentiate the chromatin, and to obtain fixed pictures of stages that pass like a vision in the living egg. A proper correlation of observations on the living protoplasm and the fixed and stained sections greatly enlarges the scope of each and is indispensable for thorough interpretation.

It is not my intention to enter into a discussion of the various theories of the morphological composition of protoplasm; I desire only to meet one objection that will certainly occur to most cytologists, viz: as Wilson ('99) expresses it, "A continuous series of size gradations exists from the largest deutoplasm spheres down to the minutest 'granules,' and these bodies and the 'alveoli,' which form the middle terms of the series, arise in a sensibly homogeneous protoplasm." As I have already pointed out, the spherules in the protoplasm of Chætopterus grade down to the size of microsomes; it is also true that granules smaller than microsomes occur. Thus microsomes are terms in a series of *sizes*. This, however, does not prove that they may not be specific elements for the size-gradation may be interpreted in various ways. Microsomes are aggregations of molecules, and it is probable that, if we could trace their origin morphologically, smaller granules would be recognized as their precursors. It might, therefore, be better to use some other term than "microsomes" for the granules in question, but any

more specific term would be open to other objections. The word microsome is used in this paper only for a class of granules which are very numerous, very uniform in size, and with other specific characters described in the text and illustrated in the figures.

III. THE STRUCTURE OF THE EGG

1. Observations

a. Before Rupture of the Germinal Vesicle

The ovaries of Chætopterus occupy the bases of the enlarged parapodia in the posterior sexual division of the body, and have the form of convoluted tubes. The wall of the tubes is made up for the most part by the ova which extend from the lumen. The pole of the ovum farthest from the lumen may be called the free pole and that next the lumen of the ovarian tube, the attached pole. The epithelial arrangement of the ova remains until they are full grown, so that there is no difficulty in distinguishing free and attached poles at any stage. It may be stated at once that the free pole becomes the animal pole of the oösperm, and the attached pole consequently represents the vegetative pole. Thus it is possible to trace the germinal areas recognizable in the oösperm back to an early stage of the ovocyte.

By so doing the so-called period of growth of the ovogenesis assumes a new significance as a period in development, and it must be studied from this standpoint. This problem, however, will be left for later treatment, though certain broad features may be mentioned from time to time in the present paper.

The following account is based on a study of the living egg and of sections of material killed in picro-acetic acid, sublimate-acetic, Flemming's fluid and Zenker's fluid; sections from each kind of material were stained in the following ways: (1) Iron hæmatoxylin followed by orange G; (2) thionin and orange G; (3) neutral gentian (see Bensley '00). So that at least twelve different combinations were used, and all gave essentially similar results in differentiating the structures described. Thus it will not be neces-

sary to describe the results of particular methods in detail. Most of the features described were seen in the living egg and demonstrated also by experimental means.

The cytoplasm and germinal vesicle will be described separately.

1. *Cytoplasm.*—The main structural feature of the cytoplasm is its differentiation into two layers, an outer or *ectoplasmic* and an inner or *endoplasmic*.

The ectoplasm (Fig. 1) covers the free hemisphere and ends a short distance below the equator so that the endoplasm comes to

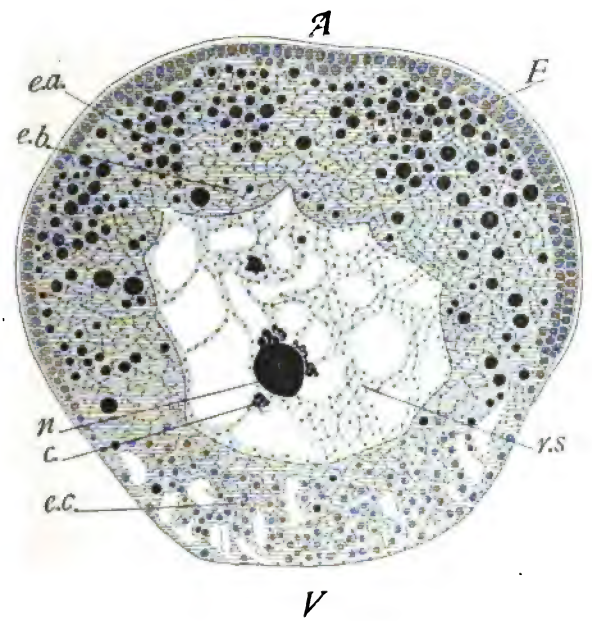

Fig. 1. Axial section of a full-grown primary ovocyte, which still formed part of the ovarian epithelium. Note that the ectoplasm is confined to the upper two-thirds of the egg.

A, Animal pole or free pole of ovarian ovocyte; *c*, chromatin; *E*, ectoplasm; *e.a.*, endoplasm *a*; *e.b.*, endoplasm *b*; *e.c.*, endoplasm *c*; *n*, nucleolus; *r.s.*, residual substance of the germinal vesicle; *s.n.*, sperm nucleus; *V*, vegetative pole or attached pole of ovarian ovocyte.

Note.—All figures of sections in this paper were drawn with the camera at a magnification of 1900 diameters (Zeiss comp. oc. 12 and obj. 2 mm. oil immersion). They have been reduced to about 0.6 of original size. Most of the drawings of living eggs were made with the camera at a magnification of about 400 diameters; these were subsequently redrawn, somewhat enlarged, in ink.

the surface at the vegetative pole. There is sometimes a defect in the ectoplasm in the center of the free or animal pole. This is particularly obvious after staining in thionin, for the spherules remain unstained and the microsomes stain intensely blue; thus is produced the effect of a blue plug in the center of the free hemisphere. The ectoplasm is covered externally by a very delicate membrane, and the vegetative pole where the endoplasm comes to the surface is naked. The constant characteristic of the ectoplasm that enables one to follow it throughout the development is the presence of a large number of spherules of very uniform size, closely set together so as to produce the effect of a pavement when one focuses on the surface of the entire egg. These are as large as the average spherules of the endoplasm, but they differ from them in many respects: (1) In the living egg they are colorless, whereas the endoplasmic spherules are yellow in mass; (2) their position and arrangement are characteristic in each stage; (3) they always stain differently from the endoplasmic spherules; thus the osmic acid of Flemming's fluid leaves them unstained, but stains the endoplasmic spherules from gray to solid black; a section of such an egg mounted in balsam appears to have a layer of vacuoles in the ectoplasm, which close examination shows to be the ectoplasmic spherules. After iron hæmatoxylin and orange G following either picro-acetic or sublimate-acetic fixation, the ectoplasmic spherules are intensely orange, while the larger endoplasmic spherules are black; this result is easily obtained by proper extraction of the iron hæmatoxylin, which at first stains all spherules black, but which washes out of the ectoplasmic spherules long before it does from the larger endoplasmic ones; if one stops the extraction at this point, and stains in orange G a very clear differentiation of the ectoplasm is obtained. Other methods and stains differentiate the ectoplasm in different ways, but as I am unable at present to reason from the microchemical reaction to the chemical nature of the spherules involved, it seems superfluous to describe the details.

There are from one to three layers of these spherules in the ectoplasm over the upper hemisphere (Fig. 1). These are embedded in the ground substance which likewise contains micro-

somes external to the spherules as well as between and on them. The layer of the ground substance external to the ectoplasmic spherules may be called the surface pellicle; during the maturation and cleavage this pellicle forms characteristic waves described later on.

The endoplasm is not so uniform in its composition as the ectoplasm. There is, in the first place, a striking difference between the upper and lower hemispheres (Fig. 1). In the upper hemisphere the protoplasm is dense, and contains an aggregation of the largest spherules, which stain black in the osmic acid or iron hematoxylin (endoplasm *a*); these are massed largely toward the periphery leaving a zone of protoplasm (endoplasm *b*); surrounding the upper half of the germinal vesicle, relatively free from the spherules. Thus thionin (which is an intense microsomal stain) shows a deep blue crescentic area surrounding the upper half of the germinal vesicle, and sending out processes among the spherules to the periphery. At the free pole these processes are strongest and may cause an interruption in the ectoplasm, where the endoplasm comes to the surface. There is a beautiful reticulum in the cytoplasm where it is not concealed by spherules, and the microsomes lie in the nodes; I have already given reasons for believing that the reticulum is an artefact. In the lower hemisphere the protoplasm is vacuolated, and contains smaller spherules that stain differently from the larger endoplasmic spherules[1] (endoplasm *c*).

Thus the endoplasm comes to the surface at two places, (1) a small area in the center of the animal pole, where the polar globules are later formed;[2] (2) over a large portion of the vegetative hemisphere where the spermatozoön usually enters.

2. *The Germinal Vesicle.*—The diameter of the germinal

[1] The endoplasmic spherules have collectively a yellow tinge that is very bright in the eggs of some individuals and relatively pale in others. The spherule-bearing endoplasm is, therefore, often named in the following pages the yellow endoplasm, or yellow substance.

[2] I have not always been able to find this ectoplasmic defect in the primary ovocyte prior to the rupture of the germinal vesicle, and at the best it is relatively slight. But I believe it to be significant, for it marks the position of a constant exposure of the endoplasm that arises at the time of formation of the first maturation spindle, and persists throughout the development. The apical flagella arise from this exposed endoplasm.

vesicle is approximately half that of the entire egg (Fig. 1), and thus comprises about one-eighth of the substance of the latter. Only a small portion of it goes to form the chromosomes of the maturation spindles. There is also a large nucleolus. The remainder is a special substance which I shall call the residual substance of the germinal vesicle (Fig. 1, *r.s.*); it plays an important rôle in the developmental processes and can be assembled, even hours after the germinal vesicle has broken down, by the action of centrifugal force; thus it is certain that it does not "intermingle" with the rest of the cytoplasm. It is important, therefore, to describe carefully the structure of the vesicle.

In the living condition one recognizes easily the membrane and the large nucleolus; the peripheral part of the vesicle contains more vacuoles than the central mass and thus appears less dense (see Figs. 6–16). Apart from the droplets occupying the vacuoles, the substance consists of an enormous number of microsomes and small spherules embedded in a homogeneous ground substance. The chromatin is not separately visible in the living germinal vesicle.

In sections stained with iron hæmatoxylin and orange G the germinal vesicle (Fig. 1) is seen to be occupied by a meshwork of a few thick strands, bounding vacuoles. The strands are penetrated through and through with microsomes that stain orange instead of hæmatoxylin like the cytomicrosomes. But when the germinal vesicle breaks down, these microsomes change their staining reaction to that of cytomicrosomes. With thionin and orange the cytomicrosomes stain an intense blue and the microsomes of the germinal vesicle, orange, until the germinal vesicle breaks down, when they, too, take the blue thionin stain. Thus the staining reaction sharply differentiates the microsomes of the cytoplasm and germinal vesicle. The membrane of the germinal vesicle consists of a thin layer of the ground substance of the nucleus in which the microsomes take the acid stain, and a thin layer of the ground substance of the cytoplasm in which the microsomes take the basic stain.

My study of the nucleolus and chromosomes is incomplete and I shall defer a detailed description for another occasion. The

close association of certain chromosomes with the nucleolus is a very striking and constant character (see Figs. 1 and 4).

It will be seen, then, that the full grown primary ovocyte is highly differentiated: (1) Its polarity is expressed by, (*a*) the constant relation of free and attached poles; (*b*) the extension of the ectoplasm over the upper hemisphere mainly; (*c*) the exposure of the endoplasm at a small spot in the free pole and over a large area at the attached pole; (*d*) the contrast between the structure of the endoplasm in the free and attached hemispheres. (2) The existence of a sharply differentiated ectoplasm, of three kinds of endoplasm, and of the various substances of the germinal vesicle, gives a degree of orginal diversity that is certainly surprising.

Our problem is to trace these substances in development, to note the appearance of other substances, and from the behavior of all in the normal and experimentally modified development, to attempt to discover their respective rôles in the formation of an embryo. We should not attempt to prejudge the matter in advance by naming certain substances yolk or food-matters and others formative substances. If these are true categories, it should be possible to demonstrate it.

The following account will show that these substances are moved by internal forces to definite locations in the embryo and become parts of definite systems of organs. It will also be shown that the localization, and part of the differentiation, is independent of the process of cell division, though closely dependent upon interaction with nuclear derivatives.

b. The Period of Maturation and Fertilization

1. *Polarization.*[1]—As long as the germinal vesicle is intact the arrangement of the various substances in the egg does not conform in all respects to the position of the future embryonic areas. The rupture of the germinal vesicle initiates a series of movements of the substances by means of which they attain to their

[1] The substances of the egg are polarized before this process, but have a radically different arrangement; a more exact term for the process would, therefore, be re-polarization, which is objectionable in other respects.

definitive positions during the prophases of the first maturation division. This rearrangement of the substances may be called the process of polarization, inasmuch as it takes place with reference to the polar axis of the egg. It produces a topographical arrangement of the substances that corresponds in many essential respects to the future embryonic areas. Thus the history of the maturation process is an important chapter in the development. Fertilization, which occurs during this period, will be considered only incidentally, as I have nothing substantially new to add to Mead's fine account.

If the eggs of Chætopterus be taken and allowed to stand in sea-water, the germinal vesicle breaks down, whether the eggs be fertilized or not; the first maturation spindle forms and moves to the predelineated animal pole; at the same time the various substances become polarized. Thus polarization is independent of fertilization. Unless the eggs be fertilized or stimulated in some other definite fashion, they remain in the metaphase of the first maturation division indefinitely (Mead '98), (Lillie '02). If the eggs be so fertilized or stimulated, the polar bodies are formed and the processes of development follow continuously.

The movements of the three classes of substances, ectoplasm, endoplasm, and the substances of the germinal vesicle will be described separately; they have reached practically their definitive positions by the time that the first maturation spindle becomes fixed at the periphery.

Ectoplasm.—Before the breaking of the germinal vesicle the ectoplasm covers the upper two-thirds of the egg; as soon as the germinal vesicle breaks down, it flows toward the vegetative pole, and, even before the first maturation spindle has become fixed at the animal pole, it has completely overflowed it (Figs. 2 and 4), thus covering up the exposed endoplasm. The membrane accompanies the ectoplasm and so covers the entire egg. This movement of the ectoplasm is clearly visible in the living egg as a series of waves of the transparent external pellicle; but, owing to the difficulty of orienting the living egg in this stage, I have not been able to follow the course of the waves satisfactorily. The sections, however, that enable one to trace the overflow of the vege-

tative pole, leave no doubt that they are associated with this process.

At the end of the polarization period the ectoplasm around the center of the vegetative hemisphere is as thick as anywhere else.

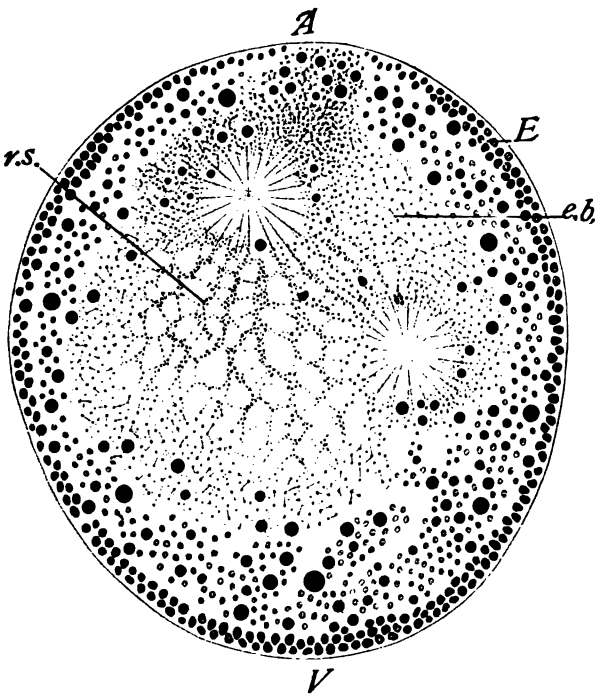

Fig. 2. Axial section through a primary ovocyte, killed about ten minutes after coming into sea-water, and three minutes after fertilization. The ectoplasm has already flowed to the vegetative pole, leaving an exposed area of endoplasm at the animal pole. Part of the *a* endoplasm has likewise flowed to the vegetative pole. The germinal vesicle has broken down and the maturation spindle is in process of formation between the two primary asters. The residual substance of the germinal vesicle is clearly seen. The chromosomes do not fall in the plane of the section. Letters same as in Fig. 1.

The original opening in the ectoplasm at the animal pole has become enlarged, and the outer end of the maturation spindle is fixed here (Figs. 2–5).

Endoplasm.—The endoplasm consists of three distinct parts as already described, (*a*) the uppermost part laden with large

spherules; (*b*) the non-spherular part overlying the germinal vesicle; (*c*) the vacuolated part containing small spherules situated below the germinal vesicle. In the process of polarization the endoplasmic substances, *a* and *b*, reverse their relations both in regard to one another and also with respect to the germinal vesicle. Substance *a* flows around substance *b* and the germinal vesicle toward the vegetative pole where it intermingles

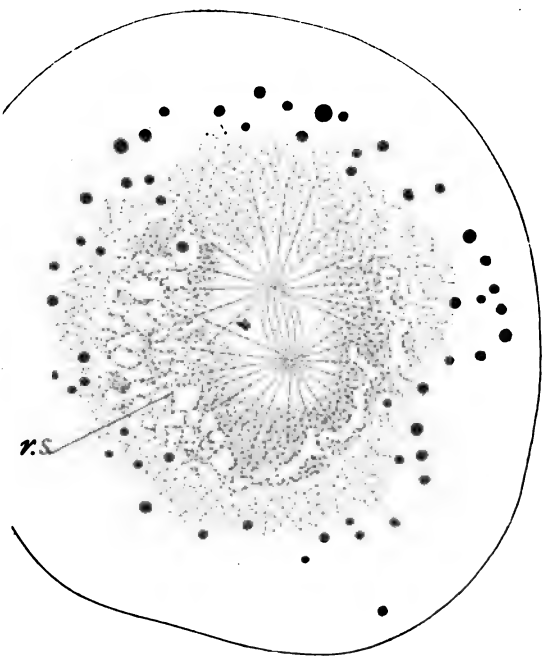

Fig. 3. A later stage of the same series, as in Fig. 2, killed twelve minutes after fertilization. It shows the entry of the first maturation spindle into the residual substance of the germinal vesicle. Orientation of section uncertain. Letters same as in Fig. 1.

with *c*, so that it is difficult for a time to distinguish them apart, except that the two kinds of spherules may be recognized intermingled (Figs. 2, 4 and 5). Substance *b* on the other hand maintains pretty nearly its original position, and the substance of the germinal vesicle passes up through it to the animal pole so as

to lie above it. The definitive positions of these substances is shown in Fig. 5. An intermediate stage is shown in Figs. 2 and 4.

Though endoplasmic substances *a* and *c* intermingle, they do not lose their identity. This is shown by their subsequent behavior; if, for instance, the eggs are allowed to remain unfer-

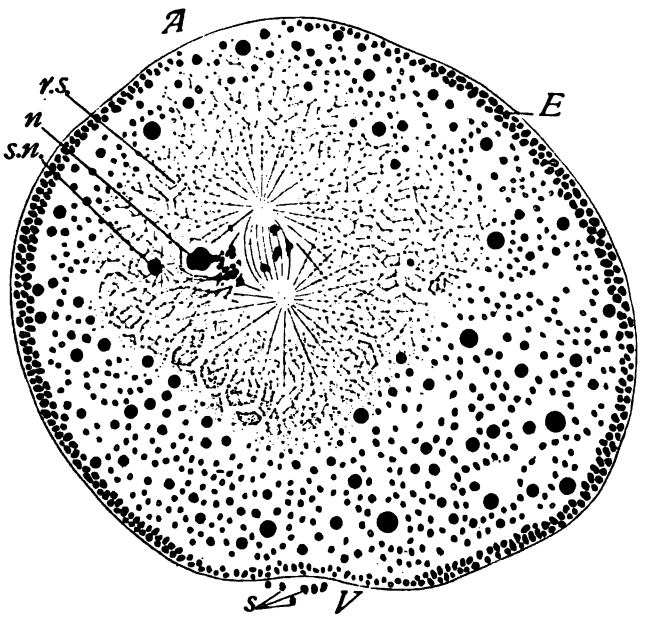

Fig. 4. Axial section of a primary ovocyte of the same series, as in Fig. 2, killed twelve minutes after fertilization. The maturation spindle is completely surrounded by the residual substance of the germinal vesicle, and the latter is directed toward the animal pole where the ectoplasm is wanting. The nucleolus has become smaller and some of the chromosomes appear to have arisen from it (compare Fig. 1). Letters same as in Fig. 1. *s*, spermatozoa.

tilized they will be found to have completely separated again in the course of about two hours (see Fig. 39, p. 208, representing a section of an egg that has stood two hours and seven minutes in sea-water). These substances also separate out in normally segmenting eggs and in unsegmented eggs that are undergoing differentiation.

The spermatozoön normally enters the egg through the exposed endoplasm of the vegetative pole, which usually appears slightly collapsed at this time (Fig. 4) and less vacuolated, as though there had been loss of fluid. That the spermatozoön may enter

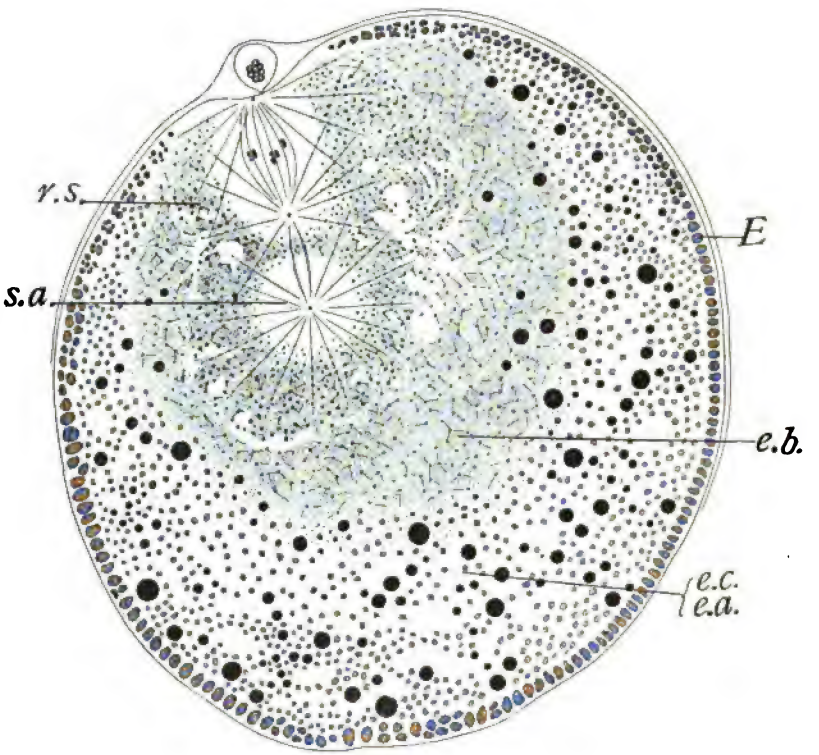

Fig. 5. Axial section of a secondary ovocyte thirty-two minutes after fertilization. *E.* Ectoplasm; *e.a.*, endoplasm *a*; *e.b.*, endoplasm *b*; *e.c.*, endoplasm *c*; *r.s.*, residual substance of the germinal vesicle; *s.a.*, sperm aster.

through the ectoplasm is shown by the fact that the eggs fertilize as readily after polarization as before.

Germinal Vesicle.—In the case of the germinal vesicle we have changes not only of position, but also in the character of the substances concerned:

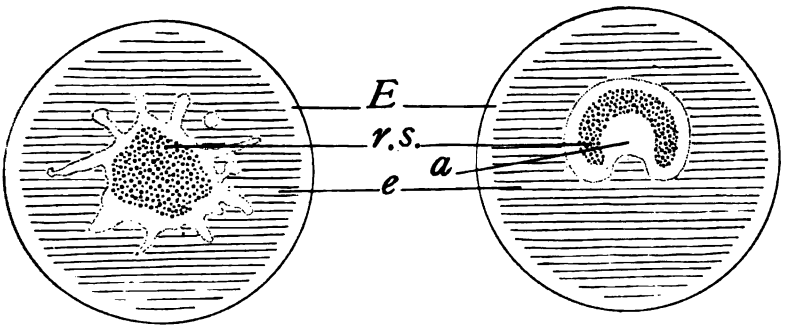

FIG. 6 FIG. 7

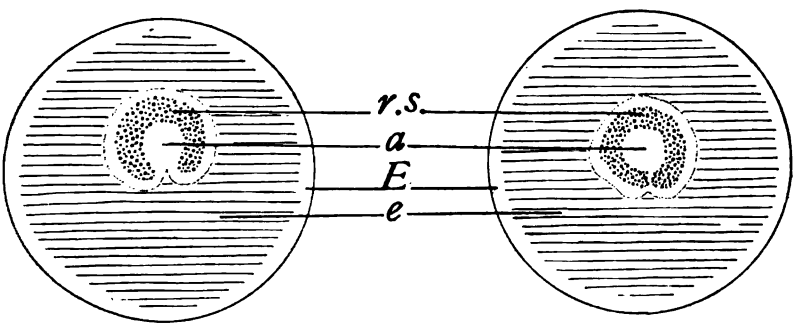

FIG. 8 FIG. 9

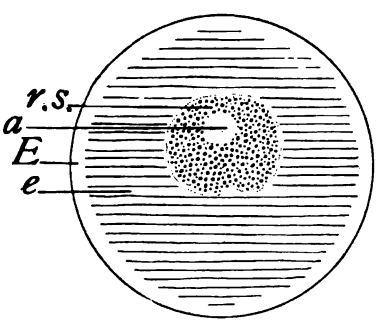

FIG. 10

Figs. 6 to 10. See text for detailed description. Behavior of substances of the germinal vesicle as seen in a single living egg. Polar view. Fig. 6. 11 A. M. Rupture of the germinal vesicle. Outflow of cortical substance of the germinal vesicle. Fig. 7. 11.04 A. M. Fig. 8. 11.07 A. M. Fig. 9. 11.15 A. M. Fig. 10. 11.20 A. M. *a*, Aster or polar view of spindle; *E*, ectoplasm; *e*, endoplasm; *r.s.*, residual substance of the germinal vesicle.

As already described, one distinguishes readily in the living germinal vesicle a clearer cortical zone and a central more granular mass (Fig. 6). Soon after the eggs are in the sea-water the membrane weakens at a number of points at once and streams of the clear cortical substance can be seen to radiate out in all directions into the surrounding protoplasm (Fig. 6); these then form little islands of clear substance surrounding the germinal vesicle, and around two of these islands radiations begin to appear, and a spindle is formed between the two asters thus arising and the latter then approach one another until they are quite near together. In a polar view one can then see the granular substance of the germinal vesicle fold around the spindle (which contains the chromosomes as sections show) and finally form a complete envelope around it and the inner aster.

Figs. 6 to 10, drawn from the living egg, illustrate this process; the entire mass is moving to the animal pole during the process. At first the residual substance of the germinal vesicle lies at one side of the spindle (Fig. 7, 11.04 A. M.) and it is surrounded by a clear zone proceeding from the original cortical layer of the germinal vesicle; the residual substance of the germinal vesicle is horseshoe shaped, and the spindle occupies the opening. Fig. 8 is a sketch of the same egg three minutes later (11.07); the limbs of the horseshoe are bending toward one another so as to enclose the spindle. Eight minutes later (Fig. 9, 11.15) the limbs of the horseshoe have almost met around the spindle. At 11.20 (Fig. 10) the limbs have fused completely and a slight indentation and corresponding projection of the endoplasm mark the place of union. The polar view of the forming spindle is always the same.

If one has a side view instead of a polar view (Figs. 11 to 16), some aspects of the process can be seen better; thus the fact that the inner end of the spindle is completely surrounded by the granular substance comes more clearly to view. Figs. 11–15 show the spindle entering the residual substance. They are drawings of a single living egg taken at about two-minute intervals (compare the section shown in Fig. 3).

Thus the germinal vesicle is transported practically intact to the animal pole; during the process a considerable quantity of fluid

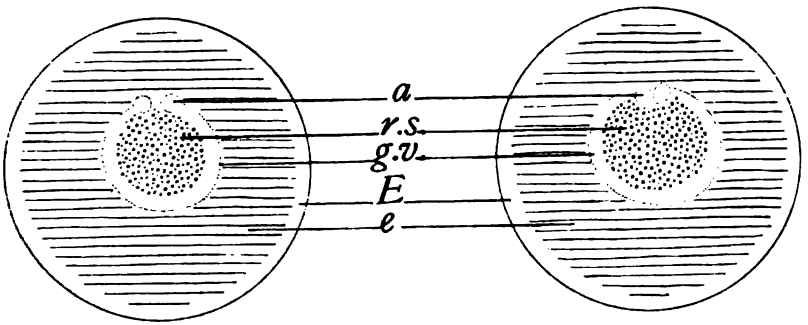

FIG. 11 FIG. 12

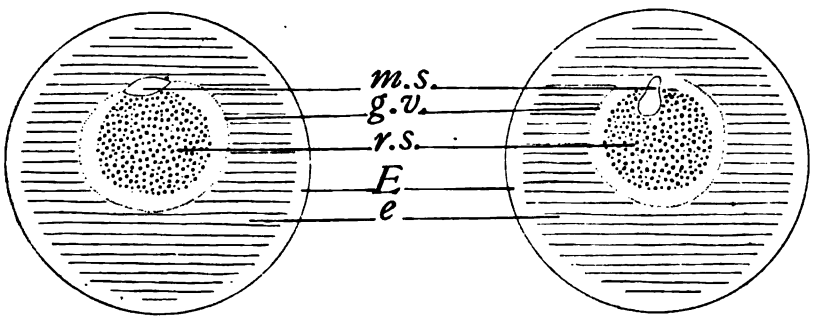

FIG. 13 FIG. 14

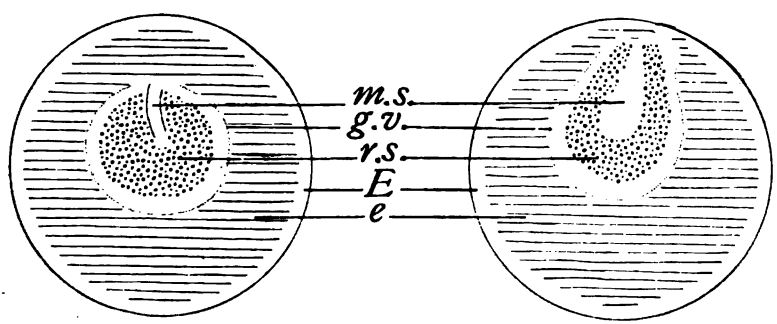

FIG. 15 FIG. 16

Figs. 11 to 16. For detailed description, see text. Behavior of substances of the germinal vesicle, side view. Figs. 11 to 15. Five views of the same living egg drawn with the camera, showing the origin of the maturation spindle and behavior of the cortical layer and residual substance of the germinal vesicle. Fig. 11. 9.46 A. M. Fig. 12. 9.48 A. M. Fig. 13. 9.51 A. M. Fig. 14. 9.53 A. M. Fig. 15. 9.54 A. M. Fig. 16. A later stage drawn from another living egg. It will be observed that the spindle arises between two separate primary asters and gradually sinks into the residual substance of the germinal vesicle, which finally forms a mantle completely surrounding it with the exception of the external end. The clear cortical layer of the germinal vesicle retains its original relation. Compare also Figs. 6 to 10. *a*, Aster; *E*, ectoplasm; *e*, endoplasm; *g.v*, germinal vesicle; *m.s.*, maturation spindle; *r.s.*, residual substance of the germinal vesicle.

has diffused throughout the cytoplasm, but this is the only loss. The end-condition is similar in some respects to the "secondary germinal vesicle" of Cyclops according to Häcker's description ('02).

The sections enable one to follow many details that are obscure in the living egg and to trace chemical transformations, obvious as changes in staining reaction in the residual substance of the germinal vesicle.

In the first place it is shown by the sections that the polarization of the spherules begins immediately after the rupture of the germinal vesicle. In the second place the chromosomes begin to separate from the surface of the nucleolus as soon as the wall of the germinal vesicle is ruptured, and the nucleolus (in consequence?) appears shrunken and vacuolated (Fig. 4). In the third place the staining reaction of the microsomes of the germinal vesicle begins to change from acid to basic, first at the periphery and later toward the center. Thus with a thionin and orange stain one finds eggs in which the peripheral microsomes of the germinal vesicle stain blue, and the central ones still stain orange; with hæmatoxylin and orange the peripheral microsomes at this stage take the hæmatoxylin and the central ones the orange. This cannot be satisfactorily shown in a figure without the use of color. This change in staining reaction rapidly embraces all the microsomes of the germinal vesicle, and at the same time the whole mass shrinks considerably owing to the diffusion of the original droplets of the germinal vesicle.

During this period of diffusion of the fluid substance of the germinal vesicle and the ensuing polarization of the ectoplasm and endoplasm, the protoplasm as a whole possesses a much higher degree of fluidity than before. The evidence for this, apart from the observed diffusion of the fluid of the germinal vesicle, is found in the change of contour of the sectioned eggs; before the rupture of the germinal vesicle, and again after the polarization is complete, the contours are perfectly regular in practically every egg. But during the period of polarization the contours are usually quite irregular, although the method of preparation is exactly the same. Moreover, the demarcation of the various endoplasmic substances is no longer clear (Figs. 2 and 4).

The strongest evidence for greater fluidity at this time is found in the fact that the ectoplasmic spherules are much more numerous and smaller than they were previously or than they are subsequently (compare Fig. 1 with 2 and 4). Evidently there is a reversible process of coagulation concerned, the spherules breaking into smaller particles as the fluidity increases, and setting or coagulating again by a process of fusion.

Mead ('98) has given a very careful account of the origin of the asters of the maturation spindle in Chætopterus. According to his account a large number of asters arise around the ruptured germinal vesicle; two of these, which he calls primary asters, become large, and form the asters and create the spindle of the maturation divisions. My observations confirm those of Mead in almost every particular; though I have not found numerous asters in the sections. Indeed in my preparations Mead's secondary asters appear simply as fluid droplets from the germinal vesicle with very slight indication of radiations. But as, according to my observations on the living egg, the primary asters likewise arise around fluid droplets from the germinal vesicle, it seems quite possible that Mead's interpretation, that there is no essential difference between primary and secondary asters, is correct. The primary asters certainly arise separately; the observations on the living egg and on sections are perfectly conclusive in this respect. They may at first be near together or very far apart, but they soon exert an influence on one another and a spindle arises between them (Figs. 11 and 12).

Figs. 1, 2, 4 and 5 illustrate sufficiently well for our present purpose the history and composition of the chromosomes. They show at the same time the microscopic structure of the residual substance of the germinal vesicle as it appears in sections, and confirm the observations of its migration to the animal pole along with the spindle. Thus Fig. 3 shows the spindle entering the residual substance; Fig. 4 a slightly later stage, and Fig. 5 the positions, structure and relations of the spindle and residual substance after they have attained the pole. The microsomes are distinguishable from ordinary cytomicrosomes only by their slightly larger size. Before the germinal vesicle breaks down the residual substance is represented by the coarse reticulum of the

germinal vesicle studded with innumerable microsomes that take the acid stain. When the germinal vesicle breaks down the reticulum shrinks together with the partial escape of the contained fluid and the staining reaction of the microsomes changes to basic like the cytoplasmic microsomes; all the fluid, however, is not squeezed out and it retains fluid droplets of considerable size that give it a permanent reticular structure. The meshes are thick and the microsomes are larger than the cytomicrosomes. It can now be traced by virtue of these structural peculiarities.

The nucleolus is carried along, with the chromosomes that are attached to it, by the residual substance, and it is therefore found at one side of the equatorial plate of the first maturation spindle (Fig. 4), where it has been figured by Mead. It gradually disappears and is lost from view.

The topography of the polarized ovocyte is shown in Fig. 5. It will be seen by comparison with Fig. 1 what a complete rearrangement of the substances of the unpolarized ovocyte has taken place; however, no new substances have been formed, though the substances of the germinal vesicle have undergone considerable changes.

2. *The Later Processes of Maturation and Fertilization.*—The formation of the polar globules and the presence of the sperm nucleus in the egg do not involve any important changes in topography of the substances, though the retreat of the egg nucleus to the center of the egg after the formation of the second polar body involves a more central localization of the residual substance of the germinal vesicle. Thus the topography of the oösperm is essentially the same as that of the polarized ovocyte.

The problems of the nature of the maturation divisions, behavior of the sperm and egg nuclei and origin of the cleavage centrosomes do not fall within the scope of this paper. So far as my observations go they confirm in most respects the account of A. D. Mead ('98). I expect to take up the behavior of the chromatin in a subsequent paper. It should be noted here, however, as bearing on the later portions of the present paper that the number of chromosomes in the maturation spindle is constantly nine, arranged usually in the form of a circle of eight with one central chromosome; the same number was found by Mead.

c. Action of Centrifugal Force in the Unsegmented Egg[1]

If the eggs of Chætopterus be taken and allowed to stand in sea-water without fertilization the germinal vesicle breaks down, the substances become polarized as already described, and the first maturation spindle is formed and moves to the animal pole, where it remains in metaphase indefinitely (unless the egg be fertilized or stimulated in some definite fashion). This condition is reached in from fifteen to thirty minutes, depending on temperature and some unknown factors.

If, at any later time before their death, the eggs be submitted to a fairly strong centrifugal action (1500 to 2000 revolutions in one minute) and then examined, it will be found that the endoplasm is arranged in three distinct layers, viz: a small gray cap, a clear band and a yellow hemisphere (Photographs A–H, and Figs. 17–22). The gray cap and clear band together take up about one hemisphere. The ectoplasmic layer is not visibly affected (see photographs and Fig. 24) and forms, as before, a continuous layer with an aperture at the animal pole. The maturation spindle is usually found fixed at the periphery immediately after centrifuging, so that it is obvious that it has not been moved by the centrifugal force.

These aggregations of substances bear no definite relation to the polarity of the egg, for the maturation spindle may be found either in the gray cap, which is then ring-shaped (Photograph H and Fig. 19), or in the center of the yellow hemisphere, or in any intermediate position. It is, however, most frequently found in the hemisphere containing the gray cap.

If now the eggs be fertilized, the polar bodies are formed at the original animal pole, and thus may appear in the gray cap, or at the center of the yellow hemisphere, or in any intermediate position; though in this case, again, they are found more frequently in the hemisphere containing the gray cap than in the yellow hemisphere (see various conditions illustrated in Photographs A–H and in Figs. 17–22).

[1] The use of the centrifuge for the purpose of studying the composition of the protoplasm was suggested to me by Dr. E. P. Lyon, who had already obtained results, about to be published, on the ova of sea-urchins by this method.

Figs. 17 to 23. Showing the effects of centrifuging upon the living unsegmented egg. Compare Photographs A–I. *E*, Ectoplasm; *e*, yellow endoplasm; *c.b.*, clear band; *g.c.*, gray cap; *g.v.* germinal vesicle; *n*, nucleolus; *p.l.* polar lobe.

Fig. 17. Eggs in sea-water at 8.55 A. M. Centrifuged at 10.45 and fertilized. Drawn with camera at 12 o'clock noon. The gray cap lies in the vegetative hemisphere.

Fig 18. Eggs in sea-water at 1.55 P. M. Centrifuged 2.30 P. M. Fertilized 2.45 P. M. Drawn 3.40 P.M. The gray cap extends into the polar lobe. Beginning of the first cleavage.

Fig. 19. Eggs in sea-water 8.50 A. M. Centrifuged 9.40 A. M. Fertilized 9.45 A. M. Drawn 9.55 A. M. The gray cap surrounds the maturation spindle and is thus ring-shaped.

Fig. 20. Eggs in sea-water 1.55 P. M. Fertilized 2.03 P. M. Centrifuged 2.50 P. M. Drawn 3.00 P. M. The gray cap has passed entirely into the larger cell *CD*.

Fig. 21. Eggs in sea-water 8.50 A. M. Centrifuged 9.40 A. M. Fertilized 9.45 A. M. Drawn 10.20 A. M. The gray cap lies opposite to the animal pole.

Fig. 22. Drawn 10.29 A. M. History otherwise the same as No. 21. Shows intermediate position of gray cap.

Fig. 23. In sea-water 9.08 A. M. Centrifuged at same time before rupture of the germinal vesicle. No stratification. Note the position of the nucleolus within the germinal vesicle.

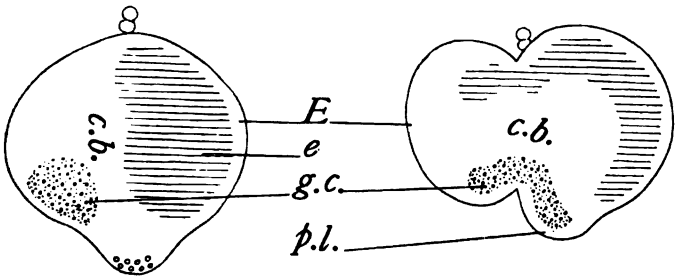

Fig. 17 Fig. 18

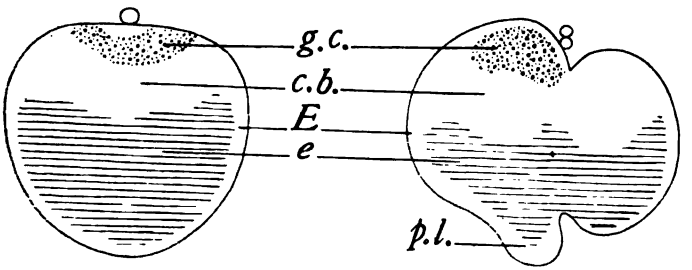

Fig. 19 Fig. 20

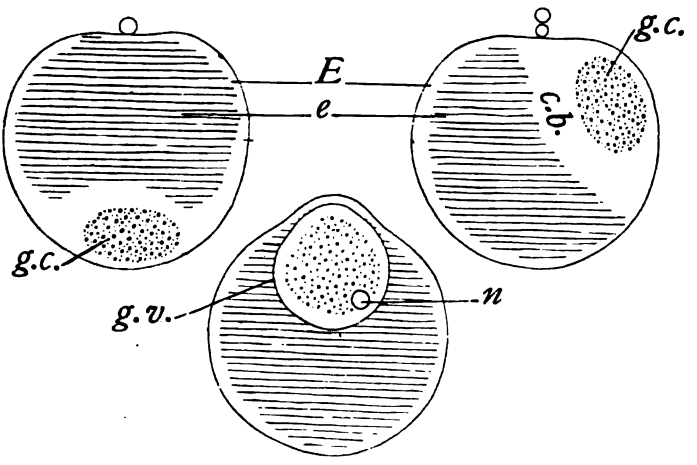

Fig. 21 Fig. 23 Fig. 22

The polar lobe appears at the usual time preceding the first cleavage, and always lies opposite to the polar globules, whatever be the position of the yellow substance (Photograph G and Figs. 17, 18 and 20). Thus, if the gray cap lies at the animal pole, the yellow substance extends into the base of the polar lobe, as normally; or, in the opposite case, the polar bodies lying at the center of the yellow hemisphere (Fig. 21), the gray cap may extend into the base of the polar lobe (Fig. 18). The structure of the peripheral part of the polar lobe is the same in either case, and does not differ from the normal.

It is therefore clear that the yellow and the gray substances are extremes as regards their specific gravity; they move freely through the ground substance of the protoplasm, and are thus distributed with reference to the direction of the centrifugal force, and so may occupy any position with reference to the polarity of the egg.

However, polarity is the force that governs the position of these substances in the normal development, causing aggregation of the yellow substance in the vegetative hemisphere and of the gray in the animal hemisphere. It is thus a stronger force than gravitation, for these substances aggregate in the same polar sense whatever be the position of the egg, but not so strong as the centrifugal force employed. It is now clear why the gray cap is thrown to the animal hemisphere more frequently than to the vegetative, for the force of polarity has already caused a partial aggregation of the substances before the centrifugal force is applied, and the eggs, therefore, tend to rotate with the heavier pole in a distal direction; but in a large percentage of the eggs, various causes combine to overcome this, the principal one being no doubt the crowding of the eggs in the bottom of the tube.

The process of cleavage takes place with reference to the polarity of the egg (Photograph I and Figs. 18 and 20) *and its fundamental form is not affected by the chance disposition of the endoplasmic substances though minor abnormalities result from certain forms of distribution.* Thus the first two cleavages are always meridional whatever the position of the yellow substance and the gray cap, and the third cleavage is equatorial and spiral in the usual sense.

The appearance of stratification given by centrifugal action is extremely striking, and it is so similiar to the banded condition described for echinoderms by Boveri ('01a and '01b) and for Dentalium by Wilson ('05), that one cannot fail to be impressed by the resemblance. Lyon has found that similar stratification may be produced in the eggs of echinoderms, annelids and tunicates by centrifugal action. So there can be but little doubt that it is a fundamental feature of egg-organization in the ova of bilateral animals.

It is a condition normally governed by the polarity of the egg, and does not become obvious under normal conditions in the egg of Chætopterus simply because the separation is not complete and precise, and thus the strata shade into one another, the animal pole being relatively light and grayish in hue and the vegetative relatively dense and yellow.

By the aid of the centrifuge one can also demonstrate that the substance of the gray cap is the residual substance of the germinal vesicle. If the eggs are centrifuged as usual immediately after they come into sea-water, the germinal vesicle being still intact, one does not get a gray cap or any banded appearance, but instead the germinal vesicle is thrown to the surface with such force that it may produce a protuberance of the cortical layer (Fig. 23), and the remainder of the endoplasm appears yellow and not strongly polarized by the centrifugal force; the cortex remains as before. The nucleolus always occupies the inner end of the germinal vesicle, showing that it is undoubtedly heavier than the remainder of the contents of the vesicle. Within the germinal vesicle there is no stratification, the granular substance preserving its earlier distributed arrangement. These eggs then mature on standing, and the maturation spindle moves to the periphery of the egg; in doing so it may take the longest course through the egg and become fixed at the pole opposite to that occupied by the germinal vesicle, or it may move to any other position. Thus it becomes practically certain that the polar force governs the migration and leads it to the animal pole. The only other assumption would be that the spindle determined polarity at whatever point on the surface it happened to be fixed, and then it would be perfectly incomprehensible why it sometimes migrates through the entire diameter of the egg.

If the eggs are allowed to stand eight to fifteen minutes in sea-water after being taken from the female, so that in some the germinal vesicle is still intact, and in others broken down, the latter always show the stratification more or less pronounced after centrifuging, and the former never show it, but instead the germinal vesicle is always peripheral and contains a granular mass similar to the substance of the gray cap.

Thus neither the gray cap nor the clear band are formed by centrifugal force unless the germinal vesicle is broken down, and the conclusion is inevitable that the entire gray cap is derived from the germinal vesicle. The yellow substance does not aggregate so closely before as after the germinal vesicle has broken down. Hence it is probable that the appearance of the clear band is due very largely to concentration of the yellow spherules at one pole. It would seem that the endoplasm has become less viscid as a result of the diffusion of substance from the germinal vesicle, so as to permit closer aggregation of the yellow granules. The structure of the gray cap completes the identification for it is the same as the residual substance of the germinal vesicle (Fig. 24; compare figures of normal maturation).

The maturation spindle is usually found fixed at the periphery immediately after centrifuging, whatever the direction of the centrifugal force may have been; when, however, it is torn loose, as sometimes happens, it is found in the clear band. Thus it is intermediate between the gray cap and yellow endoplasm in specific gravity. It is clear, therefore, that when it is situated in the region of the gray cap or yellow endoplasm, the centrifugal force must cause it to exert traction on the cortex of the ovum. That this is actually the case is shown by the form of the section of many eggs killed immediately after centrifuging, in which there is a very pronounced depression at the animal pole where the maturation spindle is attached (Fig. 24).

These sections are very interesting in two other respects: (*a*) When the spindle is torn loose from the periphery, it moves as a whole and carries the chromosomes with it—chromosomes and spindle are never found separated by the centrifugal force. This bears witness to the extreme viscosity of the spindle area, for it

can hardly be possible that chromosomes and spindle are of exactly the same specific gravity; moreover the spindle preserves its form intact, and is not bent or broken in any manner so far as I have observed, though it must be subjected to considerable strain. (*b*) If the eggs be fixed (killed) immediately after centrifuging, and sectioned, stained and mounted in the usual manner, it is found that the asters are almost entirely or entirely wanting, though sections of the same eggs prepared without centrifuging show well-developed asters at both poles of the spindle. The cytoplasm surrounding the spindle is dense and filled with microsomes more numerous and smaller than usual.

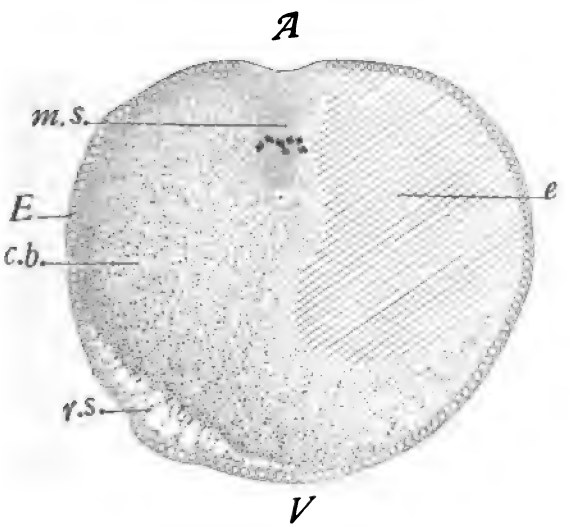

Fig. 24 Section of an egg killed in picro-sulphuric acid immediately after centrifuging. The clear band *c.b.* is seen to be densely microsomal. The ectoplasm retains its original arrangement. The yellow endoplasm and the residual substance of the germinal vesicle are at opposite sides of the egg. The maturation spindle retains its original position. *A*, Animal pole; *c.b.*, clear band; *E*, ectoplasm; *e*, endoplasm; *m.s.*, maturation spindle; *r.s.*, residual substance of the germinal vesicle; *V*, vegetative pole.

The residual substance of the germinal vesicle has been stripped off the spindle and has given place to more finely microsomal protoplasm. The sphere and centrosome, however, remain with the spindle (Fig. 24). If the eggs are permitted to stand after centri-

fuging the radiations appear; but their character is different from those of the normal egg, owing to the absence of the residual substance of the germinal vesicle around the spindle.

The most obvious interpretation of the clear band is that it represents the pure ground substance cleared of spherules by centrifugal force. This was, indeed, the interpretation that I gave it when working on the living material. The results obtained by staining, in hæmatoxylin, entire eggs killed in picro-sulphuric or picro-acetic acid, shows that this interpretation is only partially correct; for in these eggs the original clear band appears intense violet in color, and was not, for this reason, at first recognized; in such preparations the gray cap is stained relatively lightly and is seen to be highly vacuolated. The yellow mass is unstained and dense. Sections of such eggs (Fig. 24) show that the clear band contains most of the endoplasmic microsomes (which, as we have seen before, have strong affinity for basic dyes), embedded in ground substance. *Thus the centrifugal force has separated the microsomes and spherules of the endoplasm, the former then appearing as the clear band and the latter as the yellow mass.* This appears to me to furnish important evidence for the specific nature of the microsomes as argued throughout this paper. The result is very clear-cut and striking. The microsomes are thus seen to differ from the spherules in specific gravity as well as in the other respects already noted. Moreover, when the spindle is torn loose from the periphery, it is invariably found with the contained chromosomes in the clear band, thus showing that the microsomes have the same specific gravity as the chromosomes. This observation should be correlated with others described in this paper, showing origin of microsomes from chromosomes.

The definiteness and fixity of polarity is one of the most striking results brought out by centrifuging the eggs. The direction of polarity is not altered by any arrangement given to the endoplasmic substances, and it asserts its power over the maturation spindle under all the various circumstances of the experiments; similarly it determines the location of the polar lobe under all conditions, and, under normal conditions, regulates the distribution of the ectoplasm and the endoplasm.

The following statements may be made concerning the polarity of the egg of Chætopterus:

1. Polarity may be traced from the stage of the youngest ovocyte continuously.

2. In the ovocyte the free end of the egg is the animal pole and the attached end the vegetative pole.

3. It is not determined whether the oögonia are polarized or whether the polarity arises in the ovocyte owing to the relations of free and attached poles, or from some other external cause. My own view is that the polarity is inherent and is a property of the nucleus.

4. In the full-grown ovocytes and oötid the polarity is unaltered by any change of distribution of the granules of the endoplasm; it must, therefore, be a property of the residual protoplasm.

5. Polarity, however, determines the normal distribution of the granules in the egg, and is thus a force stronger than gravitation, because the distribution of the spherules is normally fixed with reference to polarity, but may bear any relation to the direction of gravitation.

6. A centrifugal force may overcome the polarity and distribute the spherules correspondingly.

The effects of polarity in the subsequent development will be onsidered in Part IV.

2. *Literature and Discussion*

a. The Axis of the Ovarian Ovocyte and its Relation to Polarity

It appears to be the rule among annelids and molluscs that the attached pole of the egg in the ovary becomes the vegetative pole of the oösperm. It is true that the number of observations bearing on this point is relatively small (Stauffacher '93, Lillie '95, Conklin '02 and '03).

On the other hand Boveri's observations ('01a and '01b) make it probable that in Strongylocentrotus the conditions are reversed. There is no theoretical objection to such a contrast. On the contrary, as Conklin has argued in his very suggestive paper on

"Inverse Symmetry" ('03), reversal of polarity would furnish a very simple and complete explanation of the condition of inverse symmetry. The considerations that Conklin brings forward in this paper, together with the actual observations in the literature, combine to render reasonably certain the hypothesis, that in all animals the axis of the epithelial ovarian ova becomes the primary axis of the segmenting ovum. It seems reasonably certain also that the poles of this axis may be interchangeable, owing probably to inverse polarization of the formative stuffs of the ovocyte.

b. The Ectoplasmic Layer

I have been surprised to find, since my observations on Chætopterus were completed, what a large number of definite observations on the existence of an ectoplasmic layer in the eggs of various animals were to be found in the literature. These have not been recently collated and their importance is, therefore, not fully realized by many embryologists. When brought together these observations bring into one category the "polar rings," "yolk-lobe" and certain other phenomena described by various authors; I therefore give rather full citations.

In Clepsine (Whitman '78) the "polar rings" evidently correspond to the two divisions of the ectoplasm of Chætopterus (see Part IV, 1, *a*, of the present paper). These rings appear after the formation of both polar globules and are situated near the upper and lower poles, respectively. Each consists of a "transparent fluid substance," radiating lines of which at first extend on the surface from each toward the equator of the egg; in my opinion these indicate the origin of the rings from an ectoplasmic layer, as in Rhynchelmis, though Whitman expresses no opinion concerning the origin of this substance. The upper ring contracts around the animal pole, but retains its central opening, which is usually eccentric, thus agreeing exactly with Chætopterus (see Photograph H). The substance of the lower ring aggregates at the lower pole in the form of a disc. Subsequently the substances of both rings plunge deep into the egg, and the upper ring substance is transmitted to the left posterior macromere, D, as in

Rhynchelmis; what becomes of the lower ring substance is uncertain.

The conditions in the egg of Rhynchelmis (Vejdovsky '88–'92) are most like those in Chætopterus. The condition before the rupture of the germinal vesicle is thus described by Vejdovsky (p. 32): "Unter der äusserst feinen Dottermembran, die man künstlich von der Eisubstanz überhaupt nicht abheben kann, erstreckt sich eine 0.003 mm. hohe Schicht des peripherischen Plasma, welches sich in Pikrocarmin intensiver färbt und deshalb ohne grosse Schwierigkeit wahrzunehmen ist (Holzschnitt Fig. 1, A). Sie besteht aus einer hyalinen Grundsubstanz, in welches äusserst feine, aber doch deutliche, in Pikrocarmin intensiv sich färbende Plasmakörnchen, eingebettet sind. Bei starken Vergrösserungen ist es nicht schwierig sicher zu stellen, dass diese Körnchen regelmässig schichtenweise und concentrisch in der Grundsubstanz angeordnet sind, in einigen Fällen scheint es aber, dass sie hier unregelmässig zerstreut sind. Diese Körnchen sind denjenigen gleichzustellen, die man viel deutlicher an den Fasern des Cytoplasmanetzes wahrnimmt. Im Leben ist diese Protoplasmaschicht braun." According to this account it would appear that the ectoplasmic layer covers the entire ovum before the rupture of the germinal vesicle.

After the formation of the polar globules the ectoplasmic layer ruptures near the equator of the egg and flows to the two poles. Cases were observed where the accumulation took place entirely at the animal pole. The defect at the animal pole produced by the maturation spindles appears to be covered up in some cases, though it is always indicated for some time by a thinner spot. Some eggs have no ectoplasmic layer, and these do not develop beyond the stage of the first cleavage spindle. The two ectoplasmic discs are transmitted entirely to the larger cell of the two-cell stage. In the four-celled stage they are confined to the large posterior macromere, and during this stage they sink into the interior and surround the sphere containing the nucleus.

The behavior of the upper accumulation of ectoplasm is thus strikingly different from the condition in Chætopterus (see IV, 1, *b*). But the transmission of the lower accumulation to the large

posterior macromere agrees with Chætopterus. Enough has been said to show what a remarkable similarity exists; the differences will no doubt be explained at some future time.

In both Clepsine and Rhynchelmis the area of the egg between the upper and lower accumulations of the ectoplasm is many times larger than in Chætopterus. This is correlated with the relatively very small size of the entoderm cells in Chætopterus. Differences in the functions of the ectoderm may also be a factor; thus all the ectodermal cells in Chætopterus are ciliated in contrast to Clepsine and Rhynchelmis where cilia are lacking. In Chætopterus the presence of ectoplasm seems to be essential for the formation of cilia.

Wilson ('04, *a*) describes an ectoplasmic layer in the egg of Dentalium; he finds that it is continuous with the "lower protoplasmic area" (substance of the polar lobe) and with the upper "protoplasmic disc." "As the egg, still unfertilized, lies in sea-water, the ectoplasm in the region of the upper disc slowly increases in amount, and in some cases this region shows a faintly radiating appearance around its periphery as if clear hyaloplasm were flowing into it from the surrounding region." It is evident that Wilson's "upper disc" corresponds to my "ectoplasmic defect" at the animal pole; the author calls "attention to the fact that the original disc is composed of very dense homogeneous protoplasm that differs markedly in character from the alveolar protoplasm of the ectoplasmic thickening that afterward extends over the whole upper surface of the egg." The resemblances between the ectoplasmic layers in Dentalium and Chætopterus will come out more clearly as the description proceeds. At present we may note as points in common (1) the "alveolar character" (Wilson) or presence of specific spherules. (2) Connection with the polar lobe and upper hemisphere. (3) Severance between the ectoplasm of the polar lobe and upper hemisphere later. (4) Existence of an ectoplasmic defect (upper protoplasmic disc of Wilson) at the animal pole. Vejdovsky's observations on Rhynchelmis agree in all these respects, and Whitman's on Clepsine in most.

Mead ('98) finds in the egg of Chætopterus "immediately inside the outer pellicle a narrow zone containing a single row of yolk-granules regularly arranged." This applies to the ovocyte of the

second order, and, at that stage, I also usually find the spherules arranged in a single layer (Fig. 15); it will be observed that Mead calls them "yolk-granules," not having recognized their true nature. Wheeler ('97) figures an ectoplasmic layer in the egg of Myzostoma; it is characterized by the presence of granules larger than microsomes, and like them staining very deeply in iron-alum hæmatoxylin. "Their number and distribution are quite variable, and they may even be entirely absent in some batches of eggs." Wheeler believes that they are chromatin granules derived from the disintegrating nuclei of nurse-cells. It is interesting to notice in his figures that the ectoplasmic layer is absent at the outer end of the maturation spindles, as in Chætopterus, Dentalium, Clepsine and Rhynchelmis. Conklin finds ('05) in the eggs of the ascidian Cynthia a "peripheral layer of deeply-staining protoplasm in which the test cells were formerly embedded and which contains no yolk, but numerous refractive spherules much smaller than those of the yolk." The layer contains yellow pigment "which seems to be associated with these small refractive spherules." As Conklin points out, Sobotta has observed and described a similar layer in the egg of Amphioxus. Conklin has traced its fate in Cynthia with the greatest exactness, and has found that it forms a yellow crescent on the posterior side of the egg, and that its substance enters into the composition of the mesoblast cells.

The existence of so similar a layer in such widely separated animals and its great morphogenic importance constitute strong reasons for believing that it is likely to be found in all the principal phyla. As will be shown in another place the assumption of its existence in certain forms, in which it has not been described, helps to explain other phenomena actually observed; for instance, the presence of the polar lobe in many mollusca, and the absence of flagella in abnormally differentiated eggs of Amphitrite (Scott) (see p. 238).

c. Residual Substance of the Germinal Vesicle

There are many observations in the literature bearing on the existence of a large quantity of residual matter derived from the

germinal vesicle after the formation of the first maturation spindle, though in most cases the observations are fragmentary and no definite interpretation is given. Figures by Wheeler ('99), Mead ('98), Coe ('99), Wilson ('04a), Vejdovsky ('88–'92) and others show a large amount of substance of the germinal vesicle remaining after the formation of the first maturation spindle; but none of these authors has traced its behavior in later stages. According to Häcker ('02) the entire substance of the germinal vesicle in Cyclops passes to the animal pole where it forms his "secondary germinal vesicle." This is like the condition in Chætopterus; but Häcker fixed his attention on the chromosomes and did not follow the residual substance farther. Without going into the details of the various accounts it is clearly shown in the literature that, as a rule, only a small proportion of the substance of the germinal vesicle enters into the formation of the first maturation spindle.

Conklin's observations ('05) are the most complete ones on the subject of the residual substance of the germinal vesicle. He finds, in Cynthia, that "as soon as the nuclear membrane has dissolved the chromosomes, nucleolus and a granular mass from which the spindle fibers are formed gather together into the center of this area of nuclear protoplasm." As the spindle forms the entire area moves to the surface of the egg, and the clear protoplasm spreads out "into a cap or peripheral layer (Ciona) or may form merely a somewhat flattened disc (Cynthia)." After fertilization the clear substance of the germinal vesicle flows to the vegetative pole, "leaving the first maturation spindle surrounded by only a small amount of protoplasm." Here it receives the sperm-nucleus and aster, and subsequently moves to the posterior side of the egg and up to the equator; "finally, after the meeting of the germ-nuclei near the posterior pole of the egg, these nuclei and the clear protoplasm surrounding them move inward to the center of the egg." "At the close of the first cleavage the nuclei and clear protoplasm move into the upper hemisphere, and thereafter, throughout development, this hemisphere contains most of the clear protoplasm and gives rise to the ectoderm." As will be seen from the subsequent description, the behavior of the residual substance in Chætopterus shows many points of similarity.

It seems probable, therefore, that the residual substance of the germinal vesicle represents a specific formative stuff of essentially the same character in different phyla.

d. Polarization

The phenomenon that I have termed polarization of the formative stuffs likewise appears to be a universal one. Here again Conklin's observations on Cynthia are the most complete; he has described definite flowing movements of the ectoplasm, residual substance of the germinal vesicle and of the endoplasm, beginning with the rupture of the germinal vesicle, by virtue of which the topography of the ovocyte is radically changed. There is first a movement of the ectoplasm and the residual substance of the germinal vesicle to the lower pole of the egg, which corresponds to the polarization described in this section. This is followed by a bilateral arrangement which corresponds to what I describe in Part IV, 1, *a*, as bilateral polarization. The rearrangement of substances in the eggs of Ctenophores described by Fischel ('03), and in Echinids described by Boveri ('01a and '01b) appears to correspond to the bilateral polarization in Chætopterus (Part IV, 1, *a*), for it does not immediately follow the rupture of the germinal vesicle but is a result of fertilization. Some of the observations of Whitman, Vejdovsky and Wilson, already considered, belong here. Many authors have described or figured a polar segregation of the yolk in the lower hemisphere following the rupture of the germinal vesicle which is undoubtedly only a part of the polarization processes taking place at this time.

Wilson, Yatsu and Zeleny have found that there is a progressive limitation of potencies of parts of the egg of nemertines beginning with the rupture of the germinal vesicle. Assuming that the various formative stuffs have limited potencies this would be the natural consequence of such a process of polarization as I have described for Chætopterus, because the new topography is more precise than the original one (compare also Conklin '05).

IV. CLEAVAGE AND DIFFERENTIATION

The problem now becomes to determine the distribution of the various substances in the early development, to follow the formation of others and to investigate the relations of all to the various forms of differentiation. To determine the normal distribution of these substances I have studied the cell-lineage up to the time of the formation of the mesoblast cell, and with the aid of sections have determined the distribution of the various substances to the different cells. The use of intra-vitam staining has likewise contributed to the study of the distribution of substances and to the identification of new ones. For the purpose of ascertaining the relations of the substances to the various forms of differentiation, I have made a renewed study of the differentiation of unsegmented eggs, which I described in an earlier paper (Lillie '02). By the action of centrifugal force an abnormal distribution of the endoplasmic substances has been produced, and the effect of this on the form of the cleavage has also been studied to a certain extent.

A statement of the more general conclusions may make the point of view of the entire description clear. In the first place the observations on cell-lineage show that the process of cleavage does not produce an essentially different distribution of substances, and that the parts of the trochophore are clearly mapped out in the segmented ovum of sixty-four cells. The topography of the unsegmented egg is, therefore, essentially similar to that of the trochophore. In the second place it will be shown that some of the substances (spherules) have in all probability a specific morphogenic value, while others appear to have simply a nutritive value, though a clear separation of the substances (spherules) into these two categories has not been possible. The morphogenic significance of the substances is shown especially well by the observations on the differentiation of unsegmented eggs, in which clear regional homologies with the trochophore are found including ectodermal, entodermal and mesodermal substances. In the third place it will be shown that the activation of these morphogenic substances is accompanied by and probably dependent upon interaction with nuclear derivatives.

1. *Formative Stuffs in the Early Normal Development*

Mead has given a good account of the cell-lineage up to sixty-four cells, and I have been able to confirm his results in practically all particulars. His description, however, was written from a different point of view, and, though complete and accurate as to the form of the cleavage, does not deal at all with the distribution of substances, which indeed he did not distinguish. It is necessary, therefore, to describe some parts in considerable detail, while for others I can rely on Mead's excellent account.

a. The First Cleavage

The period of the first cleavage is meant to include the entire time from the dissolution of the membranes of the germ-nuclei to the two-celled stage. It is characterized by the appearance of the polar lobe involving division of the ectoplasm into two parts, by the appearance of bilateral symmetry, and by formation of new substances.

1. *Polar Lobe and Division of Ectoplasm.*—At about the metaphase of the first cleavage the ovum begins to elongate in a polar direction and a slight constriction appears in an equatorial plane considerably below the equator (Photograph G and Fig. 26, p. 205); thus the ovum becomes decidedly pear-shaped, the stem of the pear being at the vegetative pole. In the region of the constriction the ectoplasm is divided so that there is a gap between the part situated below and that above it (Figs. 25 and 26); this gap is permanent, and a vegetative polar group of the ectoplasmic spherules is thus finally separated from the remainder. The constriction separating this group deepens as the first cleavage furrow begins to appear at the animal pole (Fig. 25); this furrow lies to one side of the polar globules and on the same side of the egg it passes into the constriction, so that the division becomes decidedly unequal and the polar group of ectoplasmic spherules (Fig. 25, *p.l.*), passes entirely into the larger cell (*CD*). The polar furrow deepens during the later phases of the first cleavage and thus produces a pedunculated lobe, the polar lobe, which contains all the polar ectoplasmic spherules (see Photograph I) and also

some endoplasmic spherules in its base. At the height of formation of the polar lobe the ectoplasmic waves become extremely pronounced all over it. During the completion of the first cleavage the constriction around the polar lobe gradually disappears and the substance of the polar lobe forms that part of the larger cell next the cleavage furrow, where the group of ectoplasmic spherules keeps a superficial position.

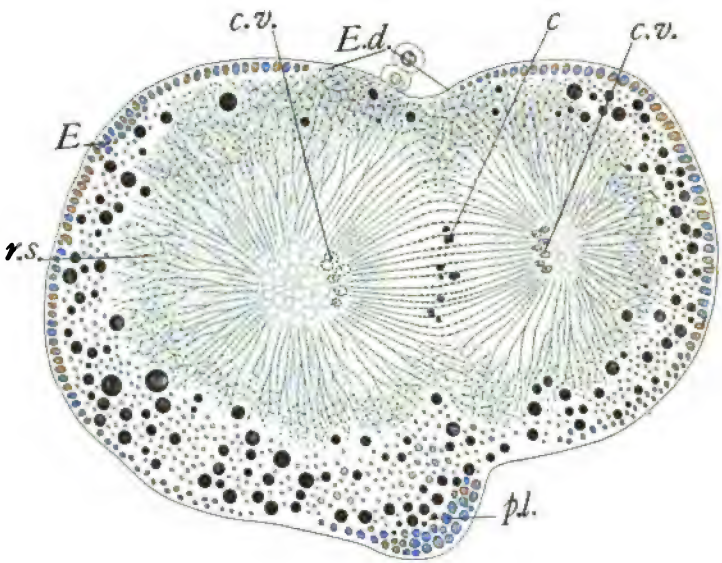

Fig. 25. Longitudinal section, first cleavage, late anaphase. Posterior end to the left, anterior to the right. The ectoplasm of the polar lobe has been separated from the remainder. *c*, chromatin masses cut off from the chromosomes; *c.v.*, chromosomal vesicles of the daughter nuclei. *E.d.*, Ectoplasmic defect; *pl.*, polar lobe; *r.s.*, residual substance of the germinal vesicle.

Mead has given a good description of the form changes of the first cleavage, and has described the polar lobe, which he calls the yolk-lobe, following the older terminology. The term polar lobe, introduced by Wilson, seems preferable to the name yolk-lobe, and I therefore employ it.

The polar lobe is fundamentally an ectoplasmic formation. The proof of this is experimental. We have seen that the endoplasmic substances may be given any arrangement with reference

to polarity by the centrifuge; but that the ectoplasm is unaffected and that the polar globules appear at the original animal pole even though this be the center of aggregation of the large endoplasmic spherules. Now the polar lobe invariably forms opposite to the polar bodies, so that in the extreme case, reversal of position of the endoplasm, the gray cap extends into the base of the polar lobe; or the protoplasm of the clear band may extend into its base in case of intermediate position of the endoplasmic substances (Photographs G and I and Figs. 17, 18 and 19). Thus the position of the polar lobe is independent of the position of the endoplasmic substances, and is determined by the polarity of the egg and the distribution, with reference to the polar axis, of the ectoplasm. Inasmuch as the animal pole is devoid of ectoplasm the ectoplasmic substance of the polar lobe may be termed briefly the polar ectoplasm.

2. *Bilateral Symmetry.*—The second striking feature of the first cleavage is the appearance of bilateral symmetry, which comes out unmistakably and permanently during the formation of the first cleavage spindle. Prior to this time I have been unable to detect any positive evidence of bilateral symmetry; thus it comes to expression in a few minutes. Like polarity itself the bilateral symmetry is independent of the distribution of the endoplasmic substances, and, in my opinion, can only be conceived as due to a force like that of polarity. The evidence for this follows:

The first cleavage spindle forms approximately in the center of the egg, but a little above the equatorial plane. Before this time the egg shows a perfect radial symmetry so far as the distribution of substances is concerned. In a horizontal section the germ-nuclei lie in the center of a mass of non-spherular protoplasm, which is surrounded by a ring of endoplasm of even thickness, and this again by an ectoplasmic ring of even thickness. As the spindle forms, however, the endoplasmic ring becomes broader at one end and narrower at the opposite end, as though the non-spherular protoplasm containing the spindle had moved toward one side of the egg; this side is the anterior, and the opposite side the posterior face of the egg, and the axis of the spindle lies in the plane of symmetry thus indicated, and therefore in the longitudinal axis

of the embryo. Thus in the early anaphase the first cleavage spindle lies eccentrically in a mass of non-spherular protoplasm, and the center of the endoplasmic spherules has been shifted toward the posterior end (Figs. 25 and 26).

At the same time the centrosphere at the posterior end becomes much larger than that at the anterior end (Fig. 26); this is very marked at the end of the prophase, and is, indeed, the first clear indication of the posterior end.

Treadwell ('97) has shown that, even when the first cleavage is equal in annelids, it bears the same relation to the axis of the embryo. But it should be noted that, when the first cleavage is unequal, as in Chætopterus, we have demonstrative evidence that the bilaterality thus established involves more than the mere determination of an axis; it involves also certain embryonic proportions of prospective significance. The cell *CD* is not only posterior in position, but it is larger and different to a certain extent in its composition, and behaves radically differently from the cell *AB;* in fact, the first cleavage predelineates the proportions and properties of the anterior and posterior ends of the embryo.

The gradual determination of the plane of bilateral symmetry furnishes a fascinating problem. It is difficult or impossible to say which of the phenomena observed to be involved are primary and which secondary; it seems probable that the primary determining factor has altogether eluded observation, and that the orientation of the spindle, the enlargement of the posterior centrosphere, and the shifting of the endoplasm and of the spindle are all consequences of some more remote cause, viz: a second process of polarization[1] analogous to that following the breaking of the germinal vesicle, but with reference to a new axis placed at right angles to the polar axis—the sagittal axis of the embryo.

[1] I have called the shifting of substances that defines the bilateral symmetry a second process of polarization because, so far as I can see, there is no immediate explanation of the phenomenon. Certainly the unequal cleavage (and the determination of bilateral symmetry that goes with it) is not due to any *chance* distribution of the substances of the egg; for on no theory of chances can one explain the uniformly normal relation of composition and of mass between the cells *AB* and *CD*.

In centrifuged eggs the first cleavage is invariably normal with relation to the polarity, and usually unequal whatever the positions of the endoplasmic substances (see Photograph I, and Figs. 18 and 19). Thus the cell *AB* may receive most, or in other cases practically none, of the endoplasmic spherules. There is certainly a tendency in the former case toward equality in size of the two cells, but the tendency toward inequality is usually markedly the stronger of the two. Thus, just as the polarity of the egg is independent of the distribution of the endoplasmic substances, so also is the bilaterality. And conversely just as polarity determines the polar distribution of these substances so does the force of bilateral polarization acting at right angles to polarity normally determine their bilateral arrangement.

This conclusion is in full agreement with my earlier opinion on the organization of the egg of Unio ('01) where I distinguished polarity and bilaterality as fundamental primitive features of the egg organization. In his fine study of the organization and cell-lineage of the Ascidian egg, Conklin ('04) likewise states his opinion that the median plane and the posterior pole are determined by the "structure of the egg" and not by external incidents such as the path of the spermatozoön. I have chosen the terms polarity and bilateral polarization as preferable because they express the opinion, which, I believe Conklin also had in mind, that they represent conditions antecedent to the distribution of substances in a polar or bilateral sense, and hence more fundamental.

We know polarity and bilateral polarization only as conditions or forces that determine definite distributions of certain substances. Beyond this they can be defined only in a negative manner; thus they are not due to any visible aggregation of unlike substances; they are not determined by the path of the spermatozoön, nor by chance location of germ-nuclei. They might be conceived to be electrical or magnetic phenomena, but all the evidence of the influence of electricity and magnetism on development runs counter to this idea. But however obscure the nature of such forces may be they are clearly demonstrated to exist (see General Discussion, "Principle of Unity").

The first division is a differential one with reference to the substance of the polar lobe, and to the amounts of other substances. Thus *CD* receives the entire area surrounding the polar globules that is devoid of ectoplasm (Fig. 29), it also receives all of the substance of the polar lobe and the larger amount of the endoplasmic spherules. On the *AB* side the ectoplasm and the endoplasmic spherules come up to the first cleavage furrow at the animal pole; whereas on the *CD* side they stop some distance from it (Fig. 29). These points are of importance with reference to the subsequent development.

3. *Residual Substance of the Germinal Vesicle and Nuclear Derivatives.*—There remain yet two features of the first cleavage to consider, viz: the fate of the residual substance of the germinal vesicle and the formation and distribution of new nuclear derivatives.

The residual substance of the germinal vesicle forms a mantle around the first cleavage spindle (Fig. 25, *r.s.*) and is thus divided approximately equally between *AB* and *CD*. After the first cleavage is complete it is difficult to distinguish it by the usual means. But by means of the centrifuge it can readily be shown that it is still a distinct substance, and that certain of its properties, at least are still the same. If the two-celled stage be centrifuged a segregation is produced in the same manner as in the unsegmented polarized egg; that is, a stratification of three substances appears in each cell—a gray cap, a clear band and a yellow mass. These may be placed in a polar direction or in any other direction. If the centrifugal force acts at right angles to the axis of the egg, the gray substance goes to the surface in the upper cell, and lies against the cleavage wall in the lower cell; while the yellow endoplasm lies against the cleavage wall in the upper cell, and against the most distal surface of the lower cell (Fig. 27). Other variations need not be described. It will be seen that it is possible in this way to demonstrate the continued separate existence of the residual substance of the germinal vesicle.

Similarly one can demonstrate it in the four-celled and eight-celled stages; Fig. 28 illustrates this for the eight-celled stage. Later cleavage stages were not centrifuged but I have other evi-

dence of its independent persistence to a later stage. In centrifuged eggs in which it passes originally entirely into one cell it is possible to follow it, on account of its massed condition, up to late cleavage stages in which its position is just internal to the nucleus. Finally, if the results of staining intra-vitam with neutral red are to be trusted, we have a method that enables one to follow it step by step with perfect accuracy into the trochophore.

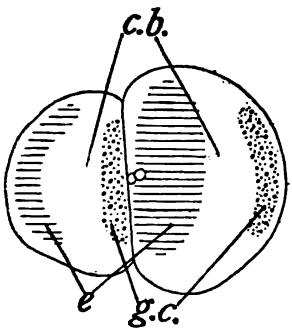

FIG. 27

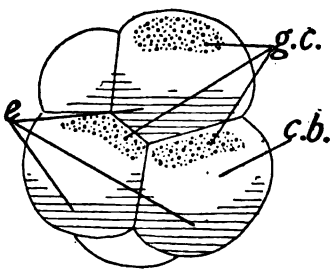

FIG. 28

Fig. 27. To show the effects of centrifuging in the two-celled stage. Drawn from the living material. *c.b.*, clear band; *e*, endoplasm; *g.c.*, gray cap.

Fig. 28. To show the effects of centrifuging in the eight-celled stage. Drawn from the living material. Explanation of letters same as Fig. 27.

That the nuclei of cleavage stages set free certain substances at each prophase is a familiar idea, that has been best set forth by Conklin. It has, however, been impossible to follow these substances far by cytological methods, so that their fate is largely a matter of conjecture. In the egg of Chætopterus such substances, "oxy-chromatin," are similarly set free, and, in addition to these, there is liberated at each mitosis a group of large granules that can be followed through at least one cell-generation in each case. These are the bodies that Mead described in the first cleavage as nucleoli.

Their history in the first cleavage is briefly as follows: They arise in connection with the chromosomes from the segmentation nucleus and separate from them in the prophase of the first division. So that the equatorial plate consists of a certain number

of chromosomes with intermingled granules. The latter are hardly larger at this time than the ends of the chromosomes, and they stain in iron-hæmatoxylin even more intensely than the chromosomes themselves. Only the chromosomes divide and in the

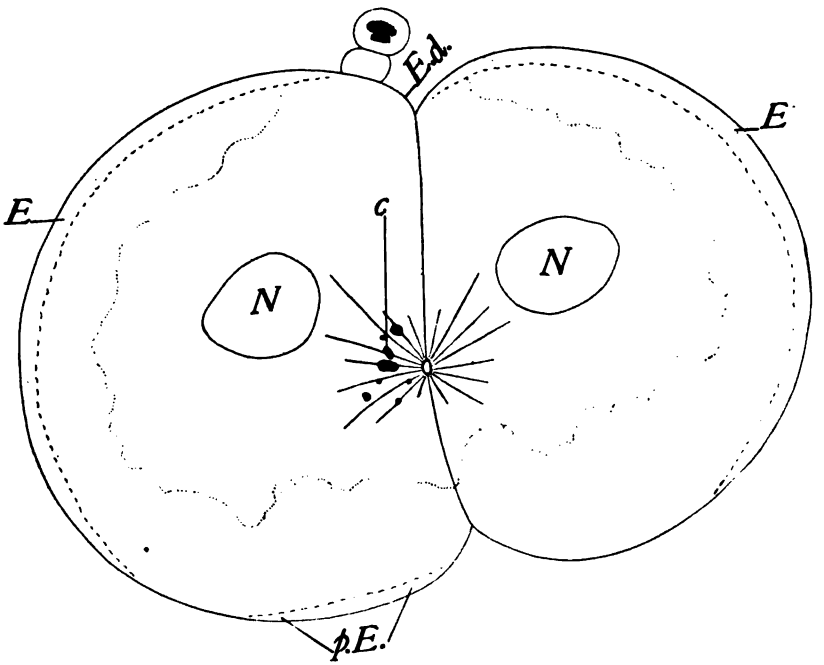

Fig. 29. Outline of longitudinal axial section of the completed two-celled stage. To show the transmission of chromatin masses cut off from the chromosomes exclusively to the larger cell. The fine dotted outline shows the boundary of the spherular endoplasm. *E*, Ectoplasm; *E.d.*, ectoplasmic defect; *c*, chromatin masses cut off from the chromosomes; *N*, nucleus; *p.E.*, polar ectoplasm.

anaphase the granules lie midway between the daughter chromosomes among the interzonal fibrils (Fig. 25, *c*). During the anaphase they become fewer and larger, no doubt by fusion with each other. In the telophase the cleavage plane passes anteriorly to them, thus leaving them invariably in the larger cell *CD*. So far as I could see, not one of them is left in the smaller cell. Fig. 29 showing the mid-body of the first cleavage is a characteristic view

of this stage, and shows with what certainty these granules may be traced into the larger cell. The origin of the bodies from the chromosomes and their differential distribution to one cell are suggestive of an important rôle in differentiation.

Thus a new group of granules arises during the first cleavage from the chromatin. A similar process is repeated in later cleavages, but whether or not it occurs to the same extent in all the cells I am unable to say. These granules attain the size of spherules by fusion; they then mingle with the *a* group of spherules of the endoplasm, and can no longer be distinguished. The repetition of this process up to sixty-four cells would produce a group of spherules staining in hæmatoxylin as numerous, probably, as the original *a* group of the endoplasm. It is possible, therefore, that spherules of the *a* group are continually disappearing and being replaced. Certain appearances in spherules of the *a* group during the cleavage favor this view; many are found that appear eroded or wasted to a crescent, a condition that I have not observed prior to the beginning of cleavage. It is impossible, therefore, to say in the late cleavage, how many of the spherules of the *a* habitus are original members of this group, and how many have been derived from the chromosomes during cleavage.

The observations are instructive as bearing on the question of differentiation of nuclei. The assumption that chromosomes may *divide* differentially has not the slightest foundation in the observed facts of karyokinesis, excepting in the maturation divisions. On the other hand the hypothesis that cytoplasmic differentiation is dependent on nuclear differentiation appears to me a necessary corollary of our growing knowledge of the characters of the chromosome complex. The above observations on differential distribution of nuclear derivatives shows a method by which not only may nuclear determination be realized, but also by which differentiation of nuclei may be obtained. If, for instance, this process of chromatin diminution takes place in certain cells and not in others, differentiation of the nuclei might result; when, on the other hand, the nuclear derivatives receive a differential distribution in daughter cells of different prospective tendencies, the problem of the determination of these cells seems simplified.

Thus nuclear determination and differentiation may exist even though every chromosomal fission be integral or equational.

I have described the first cleavage in considerable detail, because it includes part of the process of segregation of substances, particularly the bilateral polarization, and also illustrates the origin of new substances during the cleavage period. The account of the subsequent cleavages may now be divided into two parts. In the first will be given a very brief account of the form of the normal cleavage (cell-lineage) and the fate of the cells. In the second part the distribution of the substances in these cells will be traced.

b. The Form of Cleavage

The cleavage follows the usual annelid type (Figs. 30–37), *i. e.*, the ectoderm is derived from three quartets of micromeres formed by alternate dexiotropic and læotropic equatorial cleavages of the four macromeres, *A* (left anterior), *B* (right anterior), *C* (right posterior) and *D* (left posterior). The mesoblast comes from the cell *4D*; the entoderm from the cells *3A*, *3B*, *3C*, and *4D*. The somatic plate is derived from the cell *2D* or *X*.

Certain special features of the cleavage deserve particular attention. In the four-celled stage *D* is much the largest of the cells, *C* comes next, *A* and *B* are the smallest (Figs. 30 and 31). The differences between the cells *A*, *B*, and *C* are, however, relatively slight. In the second cleavage the polar lobe is indicated by a protuberance of the vegetative pole of the cell *CD* toward the left side; its material passes entirely into the cell *D*. Again in the third cleavage there is usually a protuberance at the vegetative pole of *D* (Fig. 32) indicating the location of the substance of the polar lobe, which may, therefore, be followed readily into the cell *1D*. The polar globules are attached a little posterior to the first cleavage furrow on the line of the furrow between *C* and *D*, a position that is retained throughout the cleavage (Figs. 31 and 35). The ectoplasmic defect is transmitted to the cell *1d* (Figs, 30, 33, 36 and 38).

The third cleavage is dexiotropic and approximately equal, though the ectomeres *1a*, *1b*, *1c*, and *1d* are slightly smaller than the "macromeres" *1A*, *1B*, *1C* and *1D*. Thus in the upper

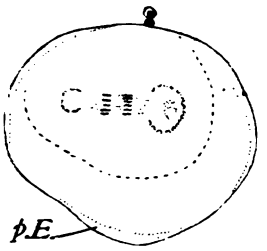

FIG. 26

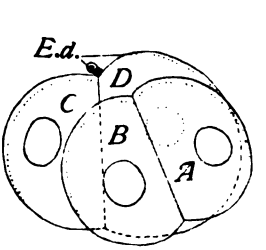

FIG. 30

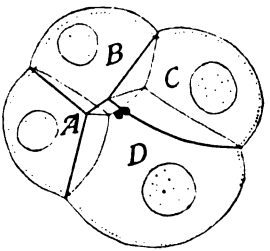

FIG. 31

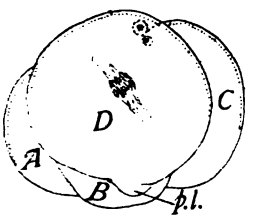

FIG. 32

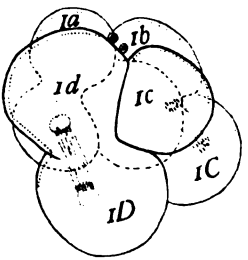

FIG. 33

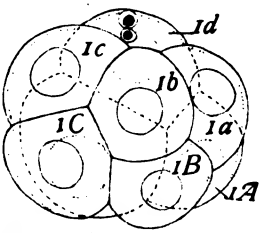

FIG. 34

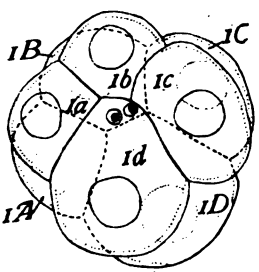

FIG. 35

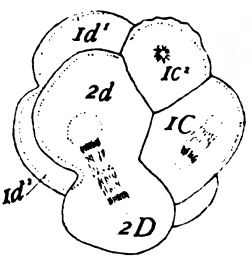

FIG. 36

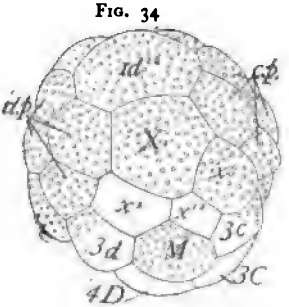

FIG. 37

Figs, 26, 30 to 37. To illustrate the form of the normal cleavage in Chætopterus. Drawn from preparations.

Fig. 26. Anaphase of first cleavage.

Fig. 30. Four-celled stage from in front. Note that the ectoplasmic defect is confined to the quadrant *D*.

Fig 31. Four-celled stage from the animal pole.

Fig. 32. Four-celled stage from behind. Early anaphase of third cleavage showing polar lobe in the *D* quadrant.

Fig. 33. Formation of the first generation of micromeres, of approximately the same size as the macromeres.

Fig. 34. Eight-celled stage from anterior end.

Fig. 35. Eight-celled stage from animal pole.

Fig. 36. Division of eight to sixteen cells to show large size of *2d*.

Fig. 37. Approximately sixty-four-celled stage seen from behind. Outline copied from Mead ('97). The small circles in the cells represent the ectoplasmic spherules (diagrammatic).

In all of the figures on this plate except the last, the ectoplasm is indicated by the dotted contour line. The relation of this line to the animal and vegetative poles should be noted.

quadrant the cell *1d* is much larger than its mates, similarly in the lower quadrant *1D* is the largest cell (Figs. 33 and 34).

In the fourth cleavage (eight cells to sixteen cells) the cell *1D* divides unequally so that its upper product *2d* is larger than the lower, the "macromere" *2D* (Fig. 36). The division of *1A*, *1B*, and *1C* are approximately equal; thus *2d* is by far the largest cell of the second quartet. In the sixteen cell-stage the cells *1d* and *2d* are much the largest cells in the egg. The orientation of the egg

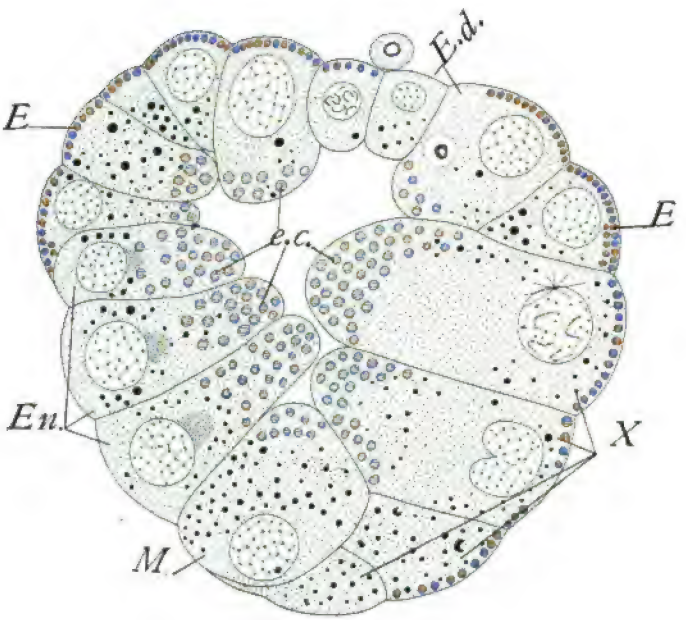

Fig. 38. A sagittal section through a stage of about sixty-four cells. The small upper cells are the apical cells. The ectoplasmic defect will be noted in the posterior apical cell to the observer's right. *E*, Ectoplasm; *e.c.*, endoplasm *c*; *En.*, endoderm cells; *E.d.*, ectoplasmic defect; *M.*, mesoblast cell. *X* derivatives of first somatoblast.

and the identification of the cells is thus a relatively simple matter. Fig. 37 (outline of cells after Mead, '97) illustrates the arrangement and prospective significance of the cells of the sixty-four cell-stage. Only the superficial part of each cell is indicated and this

is not always a good measure of relative size. The distribution of the ectoplasmic spherules is indicated by the small circles in the cells with approximate accuracy.

c. Distribution of Substances in the Cells

No cell is pure in regard to the formative stuffs it contains, but each cell, up to a late stage at least receives both ectoplasm and endoplasm, with the exception of the entoderm cells which appear to receive no ectoplasm (Fig. 38). The arrangement of the substances is the same in all ectodermal cells: (1) Externally a layer of ectoplasmic spherules; (2) the nucleus in a mass of microsomal cytoplasm; (3) just internal to the nucleus a mass of non-spherular substance; (4) within this a group of endoplasmic spherules staining black in iron hæmatoxylin (endoplasm *a*), and (5) next to the segmentation cavity a group of large spherules staining in orange G formed by segregation and fusion of the smaller endoplasmic spherules (endoplasm *c*) already described. The arrangement is similar in the mesoderm cell (though the polar ectoplasm was not distinguished in the section figured), and, with the exception of the absence of the ectoplasm, in the entoderm cells also.

This arrangement of substances is invariable; each cell is polarized; the axis of polarization of each is a radius of the egg, and the central ends are homologous. Thus each cell exhibits a stratification of substances similar to that of the entire egg.

There is, however, one difference in the arrangement of the substances in the single cells and in the entire ovum. In the latter the *c* endoplasm lies above the *a* endoplasm, whereas in the former the *c* endoplasm is most distal. It must be remembered that there is a polarization of the whole segmented egg as well as of the individual cells; the *c* and *a* endoplasmic substances retain the same relative position in the entire segmented egg as in the unsegmented. It is as though a cavity had been formed in the center of the *c* endoplasm of the unsegmented egg (Fig. 39) and nuclei placed around the periphery just within the ectoplasm, and cleavage planes cut through between nuclei to the cavity. Thus the

polarity of the cells is directly derived from the entire ovum. Nevertheless it is immanent in each cell, for, however much the arrangement of the cells may be disturbed, the arrangement of substances in the individual cells remains the same.

We have seen that the polar ectoplasm passes entirely into the *D* quadrant. Between the two and the four-celled stage it forms a polar lobe that lies at first against the *A* quadrant, but shifts to the *B* quadrant as the cells come to rest. When the spindles are formed for the third division a very small polar lobe appears in *D* (Figs.

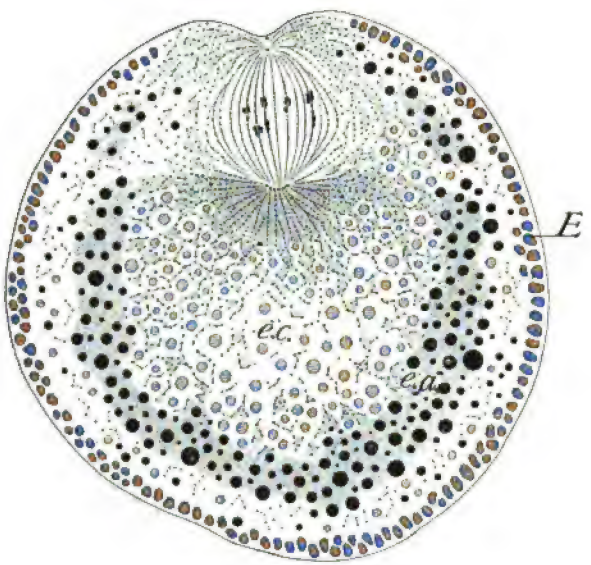

Fig. 39. Axial section of an unfertilized egg that had stood in the sea-water two hours and seven minutes before killing. The *a* and *c* spherules of the endoplasm have segregated out (*e.a.*, and *e.c.*; compare Fig. 5). A clear layer has arisen between the ectoplasm (*E*) and the *a* endoplasm; the outer centrosome of the maturation spindle has divided once, and the inner one several times.

32 and 40). Sections taken at this time show that part of the polar ectoplasm plunges into the interior of the egg (Fig. 40, *p:E.*). This is very conspicuous in the four-celled stage containing the spindles for the next division. A tongue of endoplasm then extends across, severing the portion lying internally from the portion that remains superficial in position (Fig. 40). It is possi-

ble that the upflow of the polar ectoplasm may carry some of it above the line of the next cleavage furrow, so that a certain amount may go into *1d*. An upflow of endoplasmic spherules goes on at the same time in all the cells both along the cleavage planes and also next the surface.

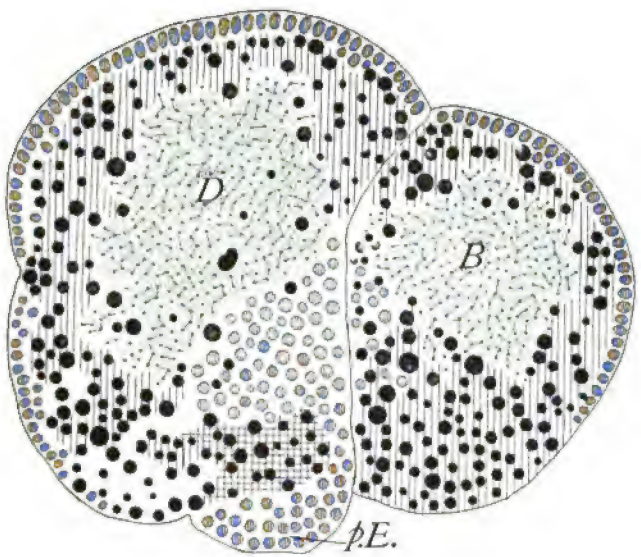

Fig. 40. Sagittal section through the cells *B* and *D* of the four-celled stage. The section passes a little to one side of the middle line; in the median section the spindles of the third cleavage were formed in metaphase, and the ectoplasmic defect was confined to *D*. The small polar lobe is confined to the *D* quadrant and contains all of the polar ectoplasm, *p.E.* Part of the latter is passing into the interior of the cell along the plane between *B* and *D*. Most of the striated spherules in the interior of the cells, however, belong to the c endoplasm, and it is impossible to distinguish exactly the boundary line between them and the polar ectoplasm.

The substance of the polar lobe (polar ectoplasm) is thus far from being pure in its distribution, for a small portion of it may pass into the cell *1d*, and the remainder is divided in two parts of which the central part is probably transmitted to the first somatoblast (*2d* or *X*), and the superficial part, to the second somatoblast (*4d* or *M*). I am not, however, perfectly certain in regard to these points, though it is probable from the position of the material; both Wilson ('04a) and Crampton ('96), moreover,

have shown experimentally that substance of the mesoblast is contained in the polar lobe.

These observations agree closely with Wilson's interesting experimental results on Dentalium; among other things he found that removal of the first polar lobe involved absence of the apical organ which normally arises in the *D* quadrant; the fact that some of the polar endoplasm probably enters into the composition of the cell *1d* might explain this curious result. Wilson, moreover, observed directly that substance of the polar lobe entered into the formation of both first and second somatoblasts.

It has been a difficult task to determine the fate of the residual substance of the germinal vesicle in the normal cell-lineage by direct observation. In the section on staining intra-vitam it will be shown that it is possible to trace it with a high degree of accuracy by this method. I will, therefore, add here only some observations made under experimental conditions that confirm the observations, made with vital staining, that this substance is distributed mainly to the first generation of micromeres: Seeing that cleavage takes place in centrifuged eggs always with reference to the original polarity and not to the induced stratification of substances, it often happens that the residual substance of the germinal vesicle, now in the form of the gray cap (Figs. 17 and 22 and Photographs), is located in the lower hemisphere. In such cases it can be seen to stream quite rapidly toward the animal pole during the process of cleavage, so that by the eight-celled stage it is usually found in the upper quartet. If, however, by any chance the third cleavage plane has come in so as to isolate it wholly or in part in the lower quartet it always comes to occupy the uppermost corner of the cells in which it is found (Figs. 41 and 42). This phenomenon may be observed with the greatest ease, because this substance in its massed condition contrasts strongly with the other cell-constituents.

There can, therefore, be no doubt of a strong inherent tendency for this substance to aggregate in the upper hemisphere. Under normal conditions it is originally located in this part of the egg, so that there is every reason to believe, on the basis of these observations alone, that normally it is distributed mainly to the

cells of the first quartet of ectomeres and their descendants. The reader is referred to the section on vital staining for more detailed evidence concerning its ultimate fate.

The distribution of the ectoplasmic spherules, excluding the polar group, corresponds exactly to the distribution of cilia (see Fig. 37). There can, therefore, be no reasonable doubt that they are specially concerned in some way with the production of cilia; this conclusion is practically *demonstrated* by the observations on the differentiation of unsegmented eggs. I would expect, therefore, to find in true prototrochal larvæ of annelids, that the distribution of the homologous substance would be confined to the prototroch, and would thus form a narrower band than in the

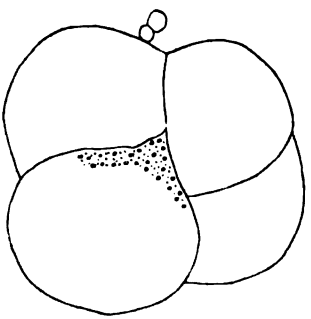

FIG. 41

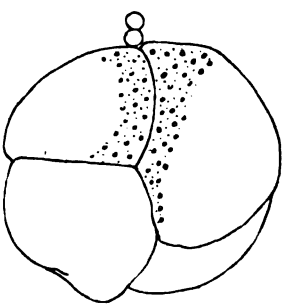

FIG. 42

Figs. 41 and 42. Two eggs centrifuged before cleavage, now in the eight-celled stage. The stippled areas represent the substance of the gray cap, which tends toward the highest point (*i.e.*, nearest to the animal pole) in the cells to which it is confined. Drawn from the living eggs.

uniformly ciliated larvæ of Chætopterus. The upper division of the ectoplasm in Rhynchelmis has an entirely different fate according to Vejdovsky: like the lower accumulation (my polar ectoplasm) it is restricted to the *D* quadrant, where it lies near the animal pole; in the four-celled stage both polar accumulations flow into the interior of the egg and surround the nucleus and periplast; subsequently the material appears to be transmitted mainly to the "mesomeres" (first and second somatoblasts).

It would appear, therefore, that the upper division of the ectoplasm has fundamentally different functions in Chætopterus and Rhynchelmis. It is possible, however, that it is really com-

posed of two substances in both forms, and that, in Chætopterus, I have overlooked the part corresponding to the main mass in Rhynchelmis, and that Vejdovsky has overlooked the part in Rhynchelmis corresponding to the main mass in Chætopterus. Seeing that the Rhynchelmis embryo is without locomotor cilia, it would be natural for the substance forming the superficial layer of the ectoderm cells of Chætopterus and associated with the formation of cilia to disappear in its phylogenic history. It is difficult to believe that substances having so similar an original disposition and structure as the upper and lower divisions of the ectoplasm in Chætopterus and Rhynchelmis are entirely different in their morphogenic functions (compare Vejdovsky's Fig. 30, Plate IV, with my Fig. 25).

Undoubtedly it would be possible by a detailed cytological study of each cell in each stage of the cleavage to follow more accurately the fate of the substances with which we have been concerned. A beginning has been made on this problem, but it has proved so difficult, that it seemed desirable to present the facts already determined, rather than delay their publication, and greatly increase the extent of this already too long publication.

The main results concerning the distribution of substances in the normal development are:

(1) The ectoplasmic defect at the animal pole is the place of formation of the apical organ with its flagella.

(2) The polar ectoplasm enters into the cells *1d*, *2d* and *4d*.

(3) The remainder of the ectoplasm forms the superficial layer of the ectoderm cells.

(4) The entoderm cells receive no ectoplasm.

(5) All cells receive some of each kind of endoplasm.

(6) New substances arise from the nuclei and are differentially distributed in some cases at least.

(7) The residual substance of the germinal vesicle is distributed mainly to the first quartet of micromeres.

d. Intra Vitam Staining

The capacity of living protoplasm to take up stains has been investigated by a large number of workers. Alfred Fischel ('99)

was the first to apply the method to embryological research; his idea was to attempt in this way to differentiate distinct elements of the ovum and if successful to follow them in the embryonic development. "Hinsichtlich des letzteren Punktes galt es mir, im besonderem, als erwünschtes Ziel, vielleicht ermitteln zu können, dass während der Furchung eine Verteilung bestimmter Elemente des Eies auf ganz bestimmte Zellen, also gewissermassen eine Teilauslese der Plasmaarten der Eizelle statthat." Fischel worked with several stains on the ova of a number of different animals, but obtained the best results with neutral red in the development of Echinus microtuberculatus. He found that stained granules appeared in a zone around the spindles and that they had such avidity for the stain, that they would completely decolorize weak solutions and become intensely red. During the resting stage of the nuclei the granules scattered throughout the cell. They were not distributed differentially in the development, but each cell contained them.

Garbowski ('04) is the only other author, so far as I know, who has applied the method to embryological work; his problem was to unite a stained portion of one egg with an unstained portion of another and to follow the subsequent development of the grafted parts. His results, however, do not bear directly on the problem under consideration. My own purpose was precisely the same as Fischel's, namely, to find if it were possible to stain constituents of the egg protoplasm intra vitam and, if so, to follow their history in the subsequent differentiation with special reference to differential distribution of such substances. *Both results were obtained; certain granules take the stain very intensely and are distributed in a very precise fashion to a particular region of the embryo.* Although several stains were used, I shall describe the results from only one, neutral red, which I found, like Fischel and Garbowski, incomparably the best for the purpose.

To obtain the results that I describe one must not use a strong solution, for then the stain becomes diffuse and the eggs do not develop far in it. The best results were obtained from eggs placed immediately after fertilization in a solution of 75 parts sea-water plus two and one-half parts of a saturated solution of neutral

red in sea-water. This solution is a very faint rose color, and the eggs develop in it perfectly normally for more then twenty-four hours. If there are many eggs in the culture they may entirely bleach the solution, all the stain being absorbed by certain gran-

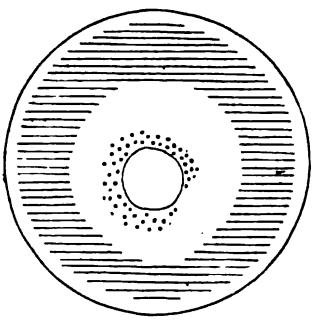

FIG. 43

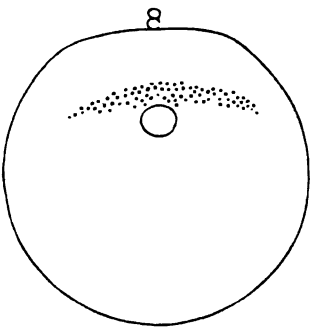

FIG 44

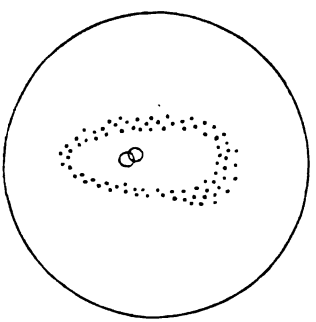

FIG. 45

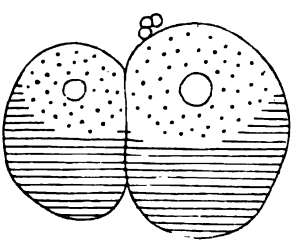

FIG. 46

Figs. 43 to 46. Drawings of living eggs reared in neutral red solution.

Fig. 43. Polar view, stage of first maturation spindle, which is represented by the central circle. The parallel lines represent the spherular endoplasm. Eggs fertilized at 10.18 A. M.; put in seventy-five parts sea-water plus two and one-half parts of a saturated solution of neutral red in sea-water at 10.20 A. M.; drawing from the living egg at 10.43 A. M. The dots around the maturation spindle represent the red-staining granules.

Fig. 44. Later stage of another egg seen from the side; the red-staining granules lie above the germ-nuclei.

Fig. 45. Stage of first cleavage spindle seen from the animal pole. The two small circles represent the polar globules. The red granules surround the spindle.

Fig. 46. Two-celled stage. Resting nuclei. Red granules scattered in the upper hemisphere.

ules that become intensely red; such granules retain the stain many hours or permanently, even if the eggs are transferred to pure sea-water. If the stain be made about twice this strength, the eggs develop abnormally after three or four hours; moreover, a diffuse pink color appears in the endoplasm also, but disappears from it very quickly after transfer to pure sea-water, though it remains in the granules that stain in more dilute solutions. Thus the stain is specific for certain granules, and the affinity of these granules for the stain is very intense.

If, then, the stain be of the proper strength one obtains invariably the following results when the eggs are put in immediately after fertilization and are allowed to remain. Up to the time of the formation of the first maturation spindle there is practically no stain in the egg, though by careful examination with an oil immersion lens one can see certain minute, scattered, bright red granules in the ectoplasm. In some cases there appears around the first maturation spindle a complete mantle of bright red granules larger than the ordinary cytomicrosomes and smaller than spherules (Fig. 43). These agree in position with the residual substance of the germinal vesicle. In other cases these granules do not stain until the second maturation spindle is formed or even later.

When the germ-nuclei are formed, these granules form a band above them and later surround them (Fig. 44). When the first cleavage spindle is formed, they form a mantle surrounding it completely (Fig. 45) and are thus divided between the two cells. As the nuclei of the two-celled stage come to rest, the red granules spread out in the upper hemisphere of each beneath the ectoplasmic spherules, *i. e.*, at the periphery of the non-spherular substance (Fig. 46).

As the nuclei elongate for the second division the red granules again accumulate around them and form a mantle to the spindles, and are thus divided between the four cells, in which, as the nuclei come to rest, they again spread out in the upper hemisphere (Fig. 47).

In the preparation for the third cleavage (four cells to eight cells) the red granules are again attracted to the nuclei and spread

out along the periphery of the spindles. But in this case the larger proportion is aggregated around the upper aster, and a relatively small number only around the lower (Fig. 48). Thus the first generation of ectomeres receives by far the larger proportion of the red granules, and this produces a most striking orientation of the egg, the upper quartet being a bright red, and the lower only faintly tinged, *except in the upper left hand corner of each macromere, where the red granules congregate after the cleavage is completed, and thus foretell* their distribution to the second quartet of ectomeres (Fig. 49). The cell *D* furnishes the most striking illustration of this on account of its large size.

I am not sure that all of the red granules remaining in the macromeres are distributed to the cells of the second quartet; but certainly the larger number are, so that very few of the original red granules remain for the third quartet.

In the later cleavage the general result is so clear as to be easily visible with the low powers; the upper half of the segmented egg has an intense red stain, the portion derived from the second quartet very little, and below this there is almost none at all.

This difference continues to increase in intensity during the formation and growth of the trochophore. At about twenty hours the exumbrella of the trochophore (Fig. 51) is brilliant, the red stain is distributed in great blotches that define a broad band interrupted dorsally and leaving only a small area at the apical pole with smaller red spots, in the center of which is the apical tuft of cilia. The subumbrella in comparison to the exumbrella is very lightly stained but the better stained specimens show a few red granules in the ectoderm that appear to define the somatoblastic plate; I am not, however, sure of the distribution of these granules in the subumbrella, which vary greatly in different cultures.

The bright red stain is practically confined to the ectoderm; the entoderm and mesoderm have at most a faint rose tinge. As the trochophore becomes older, the broad prototrochal band becomes relatively narrower, owing no doubt largely to the expansion of the head vesicle in front of it.

If now the cells of the prototroch containing the large masses

of red are examined with the oil immersion lens, it is found that the red mass is situated internal to the nucleus. Five substances may in fact be recognized in each cell: (1) the layer of ectoplasm with its characteristic spherules which are slightly stained; (2 and 3) the nuclear area containing a large vacuole; (4) the bright red mass, analyzable under the 2 mm. lens into a dense group of large red spherules; (5) an aggregation of yellow endoplasmic spherules (Fig. 52).

Now in a late cleavage stage it will be found that the arrangement of substances is the same with this exception, that the red is in the form of relatively small granules, and the vacuole is not yet formed (Fig. 50). From which it follows, either that the red granules have grown to the size of spherules, or that agglutination and fusion have taken place among them. The origin of spherules from smaller granules is thus demonstrated.

The question now arises, what is the origin of these red-staining granules that have so precise a distribution in the development. One thinks at once of the residual substance of the germinal vesicle with which the aggregation of red granules has the following points in common: (1) the original distribution around the maturation and the first cleavage spindles; (2) the position in late cleavage just central to the nucleus; (3) the size of the granules, intermediate at first between cytomicrosomes and spherules; (4) the distribution to the upper hemisphere.

One is, however, met at once by the difficulty that the amount of these granules in the later development exceeds considerably the original amount of the residual substance of the germinal vesicle. This difficulty may, however, be met by assuming that similar granules arise from other nuclei than the germinal vesicle; there is very definite evidence for this view detailed in the account of the differentiation of unsegmented eggs.

But a more serious difficulty arises; if these red granules are indeed the residual substance of the germinal vesicle, one should find that the gray cap formed from this substance in centrifuged eggs takes the red stain. Now, this is not the case; and to save the hypothesis one can only assume that the denseness of the aggregation induced by the centrifugal force is unfavorable to the

Figs. 47 to 52. Drawings of living eggs reared in neutral red solution.

Fig. 47. Four-celled stage. Resting nuclei. Red granules scattered in upper hemisphere.

Fig. 48. Third cleavage-spindles formed. Majority of red granules accumulated around the upper half of the spindles.

Fig. 49. Eight-celled stage. Resting nuclei. Same egg as Fig. 48, twelve minutes later. Details drawn only in *D* quadrant. In *1d* the red granules are uniformly distributed; in *1D* they occupy the upper left hand corner, or region of the cell *2d*.

Fig. 50. Single cell of about the sixty-four-celled stage. The red granules are now larger and lie just internal to the nucleus.

Fig. 51. Trochophore of twenty-four hours, reared in neutral red solution, the black areas show the distribution of the stain. The largest areas in three irregular rows are in the prototroch. As the larva rotated, the dorsal interruption of the prototroch came repeatedly into view.

Fig. 52. A single prototrochal cell drawn with oil immersion lens in life. The red granules are now very much larger than before. *E*, Ectoplasm; *e*, endoplasm; *N*, nucleus; *V*, vacuole.

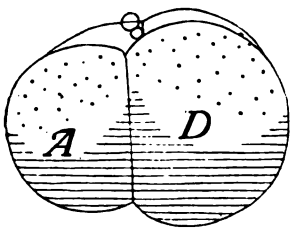

FIG. 47

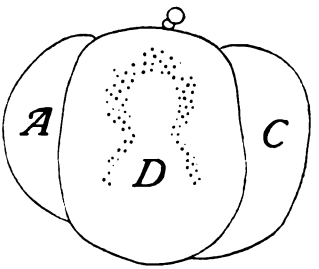

FIG. 48

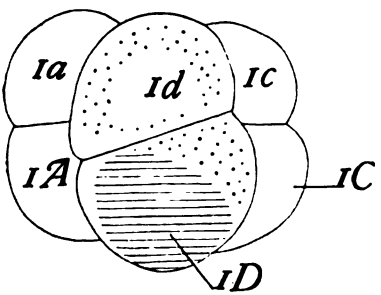

FIG. 49

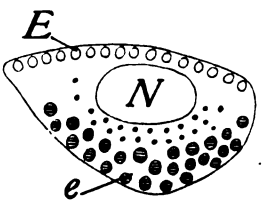

FIG. 50

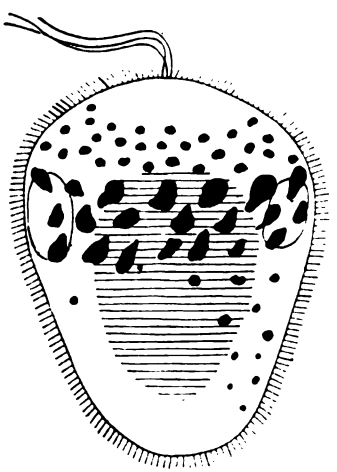

FIG. 51

FIG. 52

penetration of the stain. What one does observe is that similar scattered red granules appear on the surface of the clear band, and that these become more abundant in the upper part, and spread over the entire animal hemisphere, whatever be the position of the gray and yellow masses, as in the normal development. Now the clear band always lies against the gray cap, and, as this gradually dissolves and spreads out, it seems probable that the red granules appearing on the clear band are derived from the gray cap. It would thus appear that the residual substance of the germinal vesicle does not consist entirely of erythrophilous granules; but it is probable that the original red granules are derived from this substance.

This is at any rate the conclusion to which I have come. It should be possible to observe this without any uncertainty in the living egg, if the hypothesis is correct; but when I began to realize the significance of the question the season had passed; and I have to rely on the observations made before I realized that the gray cap was the residual substance of the germinal vesicle.

I expect to return to this question another season; but I have little doubt of the correctness of this identification of the red granules distributed to the exumbrella with part of the residual substance of the germinal vesicle and other nuclear derivatives. The resemblance in position, size of granules, and general behavior is too precise to leave much room for doubt.

2. *Differentiation Without Cleavage*

In 1902 I published a paper on Differentiation Without Cleavage in the Egg of the Annelid Chætopterus pergamentaceous, in which I showed that under certain conditions, eggs of Chætopterus might become ciliated, and exhibit other phenomena of differentiation without undergoing any process of cleavage. Similar results have been obtained for other annelid ova by Treadwell ('02) and Scott ('03 and '06). One striking fact noted in Chætopterus was that such ciliated unsegmented ova sometimes exhibited what appeared to be regional homology with the trochophore. But the structure of the ciliated ova was very

variable and such homology could not usually be demonstrated. My general conclusion at that time was that cell division is not necessary for embryonal differentiation of certain kinds; and that the usually disordered and variable character of the forms developing from unsegmented ova indicates that the essential rôle of cleavage in normal development is the localization of processes of different kinds.

However, the conclusion in no way explained how differentiation of specific cell-organs could arise in an unsegmented mass of protoplasm with only one nucleus. The most obvious inference was that the unsegmented ovum already contained substances of specific morphogenic value, that played their rôles whether distributed in cells or not. This idea has been more or less definitely in mind since the original experiments, and I, therefore, took up the subject in Chætopterus again in the summers of 1904 and 1905.

I found that a variety of methods might be employed to bring about differentiation without cleavage:

(1) By exposing the unfertilized eggs for about an hour to the action of potassium chloride in sea-water. (For details of method see Lillie '02.)

(2) By treating fertilized eggs with the same solution but in higher concentration (Lillie '02).

(3) If ova are fertilized after standing two to eight hours in sea-water a variable proportion develop without undergoing segmentation, the proportion being greater when the ova have remained longer without fertilization. Or such eggs might segment at first and the blastomeres fuse together, the subsequent development being without cleavage. The fertilization was often polyspermic in such cases.

On July 28, 1904, a batch of eggs from one female was divided into three parts, which were fertilized after standing three hours, four hours and five hours, respectively, in sea-water. None of these segmented to any considerable extent. The next morning the four-hour lot was swarming with ciliated specimens, all of which had developed without cleavage. On July 30 some eggs were divided into two lots one of which, A, was fertilized after standing in sea-water one and one-half hours and the other B,

after two and three-quarter hours. The cleavage of lot A was irregular from the start but only about 40 per cent developed without cleavage. In lot B, on the other hand, over 90 per cent failed to segment or the cells fused later on, and most of these eggs differentiated without cleavage. (For details of these experiments see the section on multinucleated, unsegmented eggs.)

(4) Fertilized eggs placed at a temperature of 10–14° C. for about twelve hours remain unsegmented and differentiate when restored to the room temperature, usually without segmentation.

In Experiment 8, 1904, two lots of eggs, A and B, were fertilized at 4.52 and 5 P. M., respectively; each was divided into two lots, A1 and A2, B1 and B2. 8.A1 and 8.B2 were placed at a temperature of about 14° C. about the time of the formation of the first polar body; 8.A2 and 8.B1 were placed at the same time at a temperature of about 16° C. When 8A.1 and 8.B2 were examined the next morning at about 9 A. M. they were unsegmented and they showed almost as sharp a segregation of the residual substance of the germinal vesicle as could be produced with the centrifuge. The preparations show that the maturation spindle remained unchanged in many cases and degenerated in others. In 8.A2 and 8.B1 there were two classes of eggs, viz: young normal larvæ and eggs like 8.A1; clearly the temperature in this case was near the lower margin of the temperature range for cleavage. A large proportion of the unsegmented eggs then underwent differentiation without cleavage at a very rapid rate.

(5) I have some evidence that an abnormally high temperature may produce the same effect (only one experiment not quite conclusive, as differentiation was not followed to a period of formation of cilia).

Careful examination of this mode of development, however, produced, showed, what I had entirely missed before, that the differentiation proceeds by the segregation and differentiation of the substances described in the preceding parts of this paper, but which I had not recognized at the time of my first study.

In describing the phenomena of differentiation without cleavage I shall leave out of account a good many accompanying phenomena which are not directly related to the problem in hand and which,

moreover, were sufficiently noticed in the earlier account (Lillie '02), such as the ameboid movements occurring at various stages of the process, and the more or less abnormal cleavage of a variable proportion of eggs in each experiment.

The unsegmented eggs that undergo differentiation may be divided into two classes according as they are *uninucleated or multinucleated;* the former constitute the type in the KCl cultures and are relatively rare in the fertilized cultures; the latter may owe the multinucleated condition either to polyspermy or to an original cleavage which is subsequently lost by fusion of the blastomeres. The multinucleated unsegmented eggs differentiate nearly as rapidly as normal ova (cilia formed in six or seven hours) whereas the uninucleated ova differentiate more slowly (cilia formed in eight to nine hours).

a. Uninucleated Ova

The conditions are simpler in the uninucleated ova in some respects and the records more complete; they may, therefore, be considered first. The process of differentiation depends on segregation of the cytoplasmic materials already described, growth of the nucleus to an enormous size, interaction of nuclear derivatives and cytoplasm, and a final rearrangement and differentiation of substances. Among the many variable phenomena, these are constant, and may, therefore, be considered as primary.

1. Early Segregation of Formative Stuffs.—The original segregation of material, whether the eggs be fertilized or not, is like the normal up to the period preceding the first cleavage; the polar globules may or may not be formed (Lillie '02); the polar lobe may appear and is then retracted; but cleavage does not take place. Then the egg-nucleus together with the nonspherular protoplasm begins to sink in toward the center of the egg. At the same time endoplasmic spherules slowly arise toward the animal pole, as in the normal development, between the nonspherular protoplasm and the ectoplasm (Fig. 53). The ectoplasm also begins to flow towards the animal pole and is soon aggregated mainly in the upper hemisphere (Figs. 54 and 55); the polar ectoplasm remains at the vegetative pole (Fig. 55). The original opening in the ectoplasm

at the animal pole may persist and the spherular endoplasm reach the surface here (Figs. 53, 55, 56). The layer of spherular enodplasm is usually considerably thinner on one side, showing that there is a bilateral polarization of the substances like the normal. The nucleus has enlarged considerably. This occurs about one and one-half hours from the beginning of the experiment.

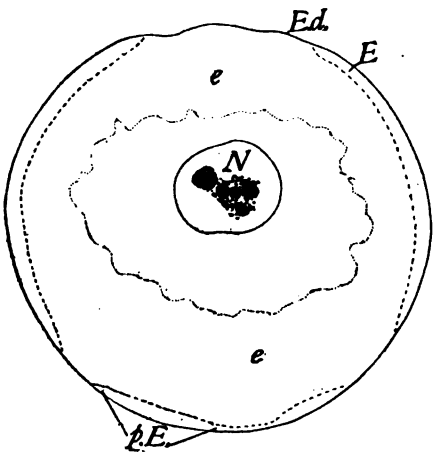

Fig. 53. Section of differentiating uninucleated unsegmented egg. *E*, Ectoplasm; *E.d.*, ectoplasmic defect; *e.*, spherule bearing endoplasm; *N*, nucleus; *p.E.*, polar ectoplasm. Eggs put in ninety-three parts sea-water plus seven parts 2½ M KCl at 9 A. M., transferred to sea-water at 10 A. M. Preserved 10.39 A. M., one hour and thirty-nine minutes from the beginning of the experiment. The dotted outline represents the boundary of the spherule-bearing endoplasm; the broken line, the ectoplasm.

The ectoplasm now flows more rapidly towards the animal pole and thus forms a relatively narrow polar ring; in some cases the opening disappears and the ectoplasm becomes heaped up in a mass (Fig. 59); as there is relatively little endoplasm in this part of the egg there appear three strata (Figs. 54, 55 and 56). This occurs about two to four hours from the beginning of the experiment. Thus in Experiment 3.1, 1904, I put some unfertilized eggs into ninety-five parts of sea-water plus five parts of 5/2 M KCl at 10.47 A. M., and the eggs were trans-

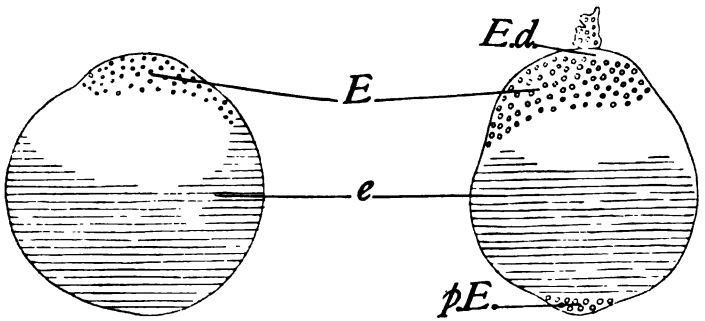

FIG. 54 FIG. 55

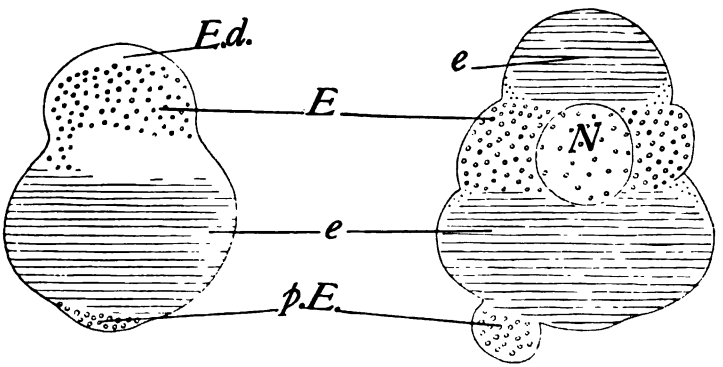

FIG. 56 FIG. 57

Figs. 54 to 58. Stages in the differentiation of uninucleated unsegmented eggs drawn from living material. *E*, Ectoplasm; *e*, endoplasm; *E.d.*, ectoplasmic defect; *p.E.*, polar ectoplasm; *r.s.* remainder of gray cap (residual substance of germinal vesicle).

Fig. 54. Eggs put in ninety-five parts sea-water plus five parts of a two and one-half M KCl solution at 3.15 P. M. Transferred to sea-water at 5 P. M. Drawn at 5.15 P. M., two hours from the beginning of the experiment.

Fig. 55. Eggs put in ninety-five parts sea-water plus five parts of a two and one-half M KCl solution at 10.47 A. M. Transferred to sea-water at 11.52 A. M. Drawn about 2.45 P. M., four hours from the beginning of the experiment.

Fig. 56. Same history as Fig. 55.

Fig. 57. Eggs put in eighty-five parts sea-water plus fifteen parts of a two and one-half M KCl solution at 11.10 A. M. Transferred to sea-water at 12 o'clock, noon. Drawn 4.12 P. M., five hours from the beginning of the experiment.

ferred to normal sea-water at 11.52 A. M., and the conditions shown in Figs. 55 and 56 were found at about 2.30 and 2.45 P. M., respectively. It will be noticed that there is quite a pronounced constriction near the equator between the unlike substances; this often becomes so deep as to produce the impression of a cleavage. The substance of the polar lobe is indicated in both cases, and in one (Fig. 55) an ameboid prominence is formed at the animal pole, owing to extrusion of endoplasm through the ectoplasmic defect occurring there.

In about half an hour to an hour more a very striking change takes place in the majority of the eggs (uninucleated, unsegmented). A mass of yellow spherules that have continued to rise toward the animal pole pushes out through the defect in the ectoplasm and forms a protuberance at the animal pole (Figs. 57, 58, and Photograph L). Thus the ectoplasm becomes a broad band encircling the ovum between the polar masses of yellow endoplasm. (For section of such a stage, see Fig. 60.) The nucleus has now become as large as the original germinal vesicle and it lies in the non-spherular protoplasm beneath the ectoplasmic band. In some cases the ectoplasmic defect at the animal pole becomes covered up (Fig. 59), and then this banded form does not occur; in such cases the ectoplasm simply becomes more massed.

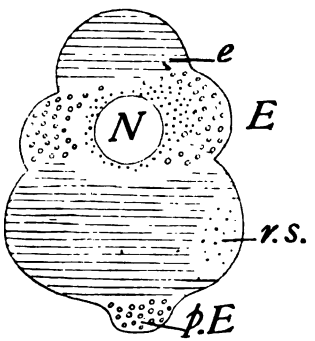

Fig. 58. Eggs taken from female at 9 A. M., centrifuged at 10.45 A. M. Fertilized 10.50 A. M. Drawn about 3.15 P. M., four hours and thirty-five minutes after fertilization.

There are some reasons for thinking that the polar masses of endoplasm are of different composition, thus it will be noticed that the original lower mass (Photograph L) affects the photographic plate less strongly than the upper mass, though to the eye they appeared alike in color.

The mere separation is also evidence for some difference in composition, though it is to be admitted that it does not constitute decisive proof. A third reason is found in the presence in the

lower mass of a small reticular area usually clearly marked at this stage (Fig. 60) and always absent in the upper mass. These facts are mentioned only as indicating that the original (or early induced) diversity of substances in the endoplasm may be greater than one would suppose. The opposed masses in this case correspond roughly to the endoplasm of the first generation of ectomeres (upper mass) and of the other cells (lower mass). The distinction is not between endoplasmic substances *a* and *c*, for these occur in each mass.

2. *Concerning the Growth of the Nucleus.*—Nuclear growth is usually accompanied by nuclear division involving the formation and division of a definite number of chromosomes at periodic intervals. Now in the eggs that we are describing the nucleus does not divide, but it grows and undergoes periodic changes that correspond to the periodic changes of dividing nuclei. Thus in any preparation the nuclei of some eggs are composed mainly of nonstaining substances, with only a few scattered stained granules similar to the "nucleoli" cast off at each normal division (Figs. 59 and 60), and other nuclei in ova on the same slide, and thus of precisely the same age and mode of preparation, are dense masses of intertwined chromosomes (Fig. 61). These conditions undoubtedly represent successive phases of nuclear activity, and demonstrate a periodic succession of chromatic and non-chromatic stages. Inasmuch as the number of chromosomes in the older and larger nuclei is much greater than in the younger and smaller, it cannot be doubted that there is a periodic formation and division of chromosomes as in the normal cleavage.

The egg shown in Fig. 61 was killed three hours and seventeen minutes after the beginning of the experiment. After making due allowance for the slower rate of development it would correspond in age to about a normal sixty-four celled stage, which would contain, if unfertilized, $9 \times 64 = 576$ chromosomes. A calculation of the chromosomes in this uninucleated egg gives a roughly corresponding number; the nucleus ran through four sections and there were considerably over 100 chromosomes in each. (All in the one section figured could not be shown in the figure.) The number corresponds so closely to the theoretical requirements,

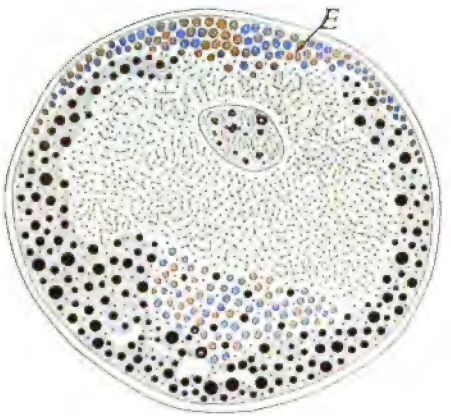

Fig. 59. History same as 53, preserved two hours and nineteen minutes from beginning of experiment. The ectoplasm aggregated in animal hemisphere; ectoplasmic defect covered up; *a* and *c* endoplasms separating out.

of which there were nine, tend to remain together. There is no other evidence for this than I have given. In any event the steady increase in number is a strong support for the individuality hypothesis.

That the nucleus does not divide under such circumstances may be attributed to the absence of centrosomes and spheres, which are usually lacking in the uninucleated unsegmented eggs in the early stages. The reason for this defect is not very clear, especially in the case of those eggs where the stimulus to development is furnished by normal fertilization. But, even when the stimulus is that it has the value of an actual demonstration of the view expressed in the last sentence of the preceding paragraph.

The arrangement of the chromosomes in Fig. 61 is suggestive; they are not uniformly distributed, but are arranged in four groups. The number of groups in the entire nucleus was at least double as many. The only explanation of such a grouping that occurs to me is contained in the hypothesis that all descendants of each original chromosome,

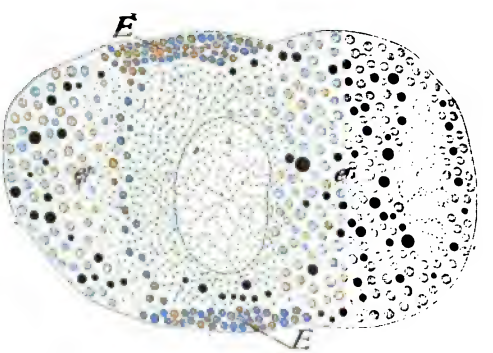

Fig. 60. History same as 53, preserved four hours and eighteen minutes from beginning of experiment. Longitudinal section of three-banded form; original animal pole probably to left. Nucleus in achromatic condition. Vacuolated area to right characteristic of eggs in this condition.

supplied by potassium chloride, certain eggs form asters and spindles, so that the potentiality of these structures must be supposed to reside in the egg protoplasm. The series of preparations upon which I rely for most of the data concerning the nuclei is from a potassium chloride series in which the polar bodies were not formed;[1] it is thus possible that, in this case, there is a connection between the omission of the process of formation of the polar bodies and the absence of asters. Such an explanation might also apply to the uninucleated, unsegmented eggs found in fertilized series; these constitute a relatively small proportion of all the eggs in such a culture, and in a normal lot of eggs some fail to fertilize, and do not form polar bodies; if we supposed that some extract of the sperm stimulated differentiation in such eggs we would get the above result.

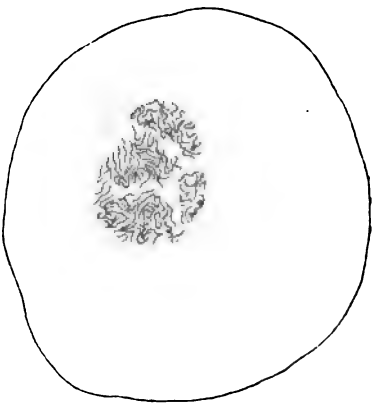

Fig. 61. History same as 53, preserved three hours and seventeen minutes from beginning of experiment. See text for description.

3. Interaction of Nucleus and Cytoplasm.—After the nucleus has reached about the size of the germinal vesicle a strong mutual attraction between the nucleus and the ectoplasm begins to be apparent, when the nucleus is in the chromatic condition. This is shown in one of two ways: Either the ectoplasm is drawn into the interior of the egg where it forms a mass in contact with the nucleus, or the chromatic part of the nucleus is drawn out to the ectoplasm. It is interesting to note that the former corresponds to the normal procedure in Clepsine (Whitman '78) and Rhynchel-

[1] If the polar bodies are not formed, the looped or elongated form of chromosome does not appear in the uninucleated, unsegmented ova until between one and two hours from the beginning of the experiment. Previous to this time one finds only irregular forms similar to the chromosomes of the maturation spindles. Thus the chromosomes apparently carry out the maturation divisions within the egg but without forming daughter nuclei, before the form of chromosome characteristic of the cleavage divisions appears.

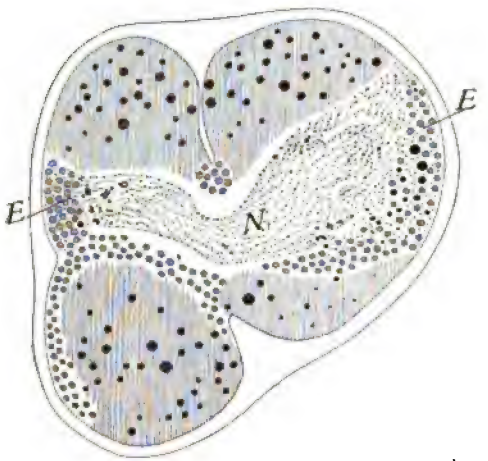

Fig. 62. Eggs put in ninety-five parts sea-water plus five parts 2½ M KCL at 11.10 A. M., five hours and ten minutes from beginning of experiment. For description see text.

mis (Vejdovsky '88–'92). When the ectoplasm has gathered in the interior it collects on one side on the large nucleus, and a very important change takes place. A large quantity of the chromatin aggregates on the margin of the ectoplasm, and, while the remainder of nucleus returns to the achromatic condition, this portion diffuses in the form of minute particles (microsomes) between the ectoplasmic spherules, which are thus completely interpenetrated by a chromatic network. Eggs in which the ectoplasm has been attracted into the interior apparently do not develop farther.

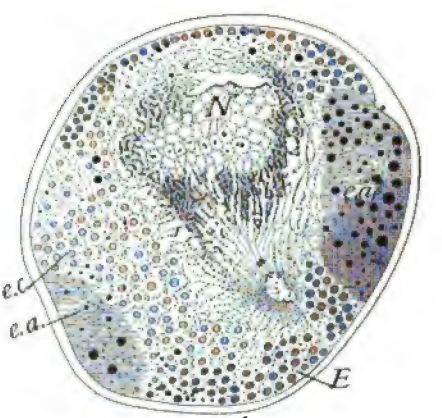

Fig. 63. History same as 62. For description see text.

When the ectoplasm remains external the chromatic part of the nucleus is drawn to it. Fig. 62 illustrates a case in which there were two accumulations of the ectoplasm on opposite sides of the egg; the attraction acting on the nucleus has drawn the latter out into a broad band extending from one mass of ectoplasm to the other. The chromatic portion of the nucleus is next the ectoplasm on each side, leaving an achromatic nuclear band in the center. The chromatin is broken up into small particles which are scattered between the ectoplasmic spherules.

A single section, such for instance as is shown in Fig. 63, shows the outline of the nucleus still intact; the chromatin in strands with large varicosities is seen stretching off toward the main mass of the ectoplasm, and the farther we trace it from the center of the nucleus the more the chromatin is broken up; radiating lines of granules can be seen extending from larger masses of chromatin in between the ectoplasmic spherules, so that all intermediate stages between masses of chromatin and scattered microsomes may be seen. Many similar sections occur on the same slides. Such granules are very much less abundant between the ectoplasmic spherules in stages preceding this.

The living material furnishes the most conclusive proof of the migration of the chromatin halo of the nucleus. If the living eggs be examined under pressure at this time, one sees a gray halo around each large nucleus (Photograph J and Fig. 58); a little later this may be seen to stretch out toward a surface aggregation of ectoplasm and to flow into it losing its identity in the larger mass (Photograph K).

The intermingling of a large part of the nucleus, distinguishable in the living condition as a gray halo and in sections as a peripheral chromatin layer, is one of the most striking and suggestive phenomena concerned in the differentiation of the uninucleated unsegmented eggs. Concerning the facts there cannot be a particle of doubt. The process can easily be observed in the living condition, and even photographed, as Photographs J and K prove. The preparations are even more conclusive, if possible, for one can study the details more leisurely. Nor is there any doubt, in my opinion, that the chromatin particles become microsomes. The gradation from larger chromatin masses to undoubted microsomes is perfect, and proceeds in an orderly direction from the nucleus outward (Figs. 62 and 63).

The interpretation of this phenomenon must proceed from the assumption that it is the result of a process occurring in the normal development, probably in each generation of cells, but which has been inhibited by the abnormal nuclear conditions, until it breaks loose by sheer force. Its striking character is due to the summation of a number of relatively inconspicuous processes of the nor-

mal development. It, therefore, furnishes to some extent a measure of the amount of chromatolysis within the same period of the normal development.

The result necessitates a more careful inquiry into the normal conditions; at present it does not seem advisable to attempt a detailed comparison. I may say, however, that the process appears to me to represent more than the formation of the chromatic granules that lie between the groups of daughter chromosomes in the normal anaphase (see description, p 202, and Fig. 25); because I believe that such granules escape into the cytoplasm periodically in the uninucleated unsegmented eggs, and do not accumulate within the nucleus. The matter is still under investigation.

Finally it should be noted that such distribution of chromatin is not limited to the ectoplasm, but occurs probably in the endoplasm also in later stages.

4. Later Distribution of Formative Stuffs.—That the ectoplasm undergoes a radical change by this intermixture of chromatin is proved by two striking phenomena: (1) The mass of chromatin-charged ectoplasm immediately overflows the remainder of the substance of the egg, usually, but not always, covering it completely; (2) the staining reaction of the ectoplasmic spherules changes radically; whereas previously they could not be stained with iron hæmatoxylin without overstaining the rest of the egg, they now take the hæmatoxylin strongly. These two phenomena will be considered separately.

Overflow (unicellular gastrulation). I observed this process first in the summer of 1904. The most complete records are in Experiments 5 and 1 of that season, from which I take the following account: Experiment 5 was started at 11.10 A. M. July 28, 1904. Division 1 of this experiment was treated as follows: The eggs were put into a mixture of ninety-five parts sea-water plus five parts 2½ M KCl at 11.10 A. M. Part a was transferred to sea-water at 12.05 P. M. (fifty-five minutes). The designation of this experiment is thus 5.1.a. At 3.20 P. M. many eggs had reached the elongated condition shown in Figs. 57 and 58 with two terminal masses of yellow endoplasm, and a broad equatorial band of ectoplasm through which the large nucleus could clearly be seen. At

5 P. M. the ectoplasmic band in many eggs had ruptured on one side transforming it into a saddle-shaped mass, and the two masses of endoplasm met and fused on the opposite side of the egg (Fig. 64). The nuclear area is situated beneath the ectoplasmic accumulation.

It is undoubtedly at this time that the chromatic matter of the nucleus intermingles with the ectoplasm, though I did not observe this in 1904 for the simple reason that the eggs were not examined under pressure. But in 1905 this phenomenon was observed as already described.

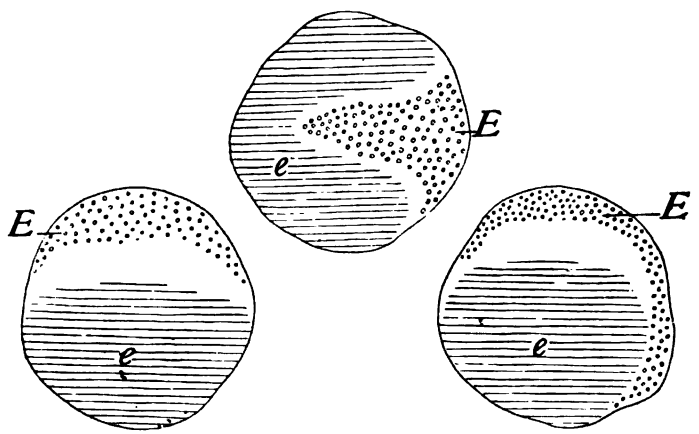

FIG. 65 FIG. 64 FIG. 66

Figs. 64 to 70. Later differentiation of uninucleated unsegmented eggs from life.

Fig. 64. Form produced by rupture of ectoplasmic band on one side. Eggs put in ninety-five parts of sea-water plus five parts 2½ M KCl at 11.10 A. M. Transferred to sea-water at 12.05 P. M.; drawn at 5 P. M., five hours from beginning of experiment.

Fig. 65. Slightly later stage than 64.

Fig. 66. Same egg as sixty-five drawn three minutes later; the ectoplasm has overflowed a large part of the endoplasm.

All stages of the rupture of the ectoplasmic band were observed; the notes read "all stages may be seen; the granular band (ectoplasm) first becomes broader on one side, then the yolk masses (endoplasm) approach and fuse and the band ruptures. Thus the yolk has the characteristic shape shown in the figure."

Then the ectoplasm accumulates in a mass (Fig. 65) and

immediately begins to spread rapidly over the surface of the egg (Figs. 66 and 67) until there is a complete surface layer of ectoplasm. The notes read "This overflow of the gray matter (ectoplasm) can easily be watched as it takes place rapidly, and all stages may be seen at once on a single slide." Two drawings of the same egg (Figs. 65 and 66) accompanying Experiment 1, 1904, show that a considerable part of the process of overflow may take place in three minutes. An uncovered spot, which probably corresponds to the position of the polar ectoplasm, may persist for some time, and the egg thus affords a striking picture, as of epibolic gastrulation in a unicellular mass of protoplasm (Fig. 67).

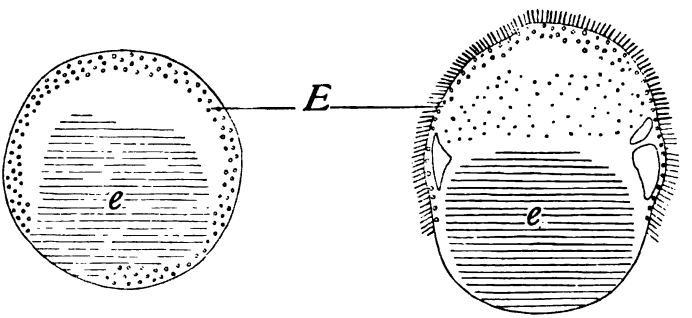

FIG. 67 FIG. 68

Fig 67. Another egg; slightly later stage. Unicellular "gastrula."

Fig. 68. Ciliated uninucleated unsegmented egg drawn about twenty-three hours from beginning of experiment. The vacuoles are about in the position of the prototroch of the larva.

In many cases the process of overflow is incomplete, or does not involve the yolk mass at all, thus leading to conditions described beyond. There is usually more or less endoplasm included in the overflowing mass; in some cases a very considerable quantity, so that the layer established by the overflow may be quite thick. This is a parallel condition to that found in the ectoderm cells normally, in which, as described on p. 207, only the layer external to the nucleus is composed of ectoplasm, and the remainder of the cell of endoplasm.

Change of Staining Reaction. Sections made for the purpose of studying the process of overgrowth show that it does not begin until the ectoplasmic spherules have been so modified by the

intermixture of chromatin that their staining reaction to iron hæmatoxylin has changed considerably. Although this approximates their stain to that of some of the endoplasmic spherules, there is not the least difficulty in distinguishing them. The ectoplasmic spherules are smaller, or at least more uniform in size, less spherical, and are massed differently; the substance in which they are embedded likewise appears different from that of the endoplasm, so that the total effect of the ectoplasm even when its spherules stain black is entirely different from that of endoplasm.

It appears as if the ectoplasmic spherules become completely impregnated with chromatin, just before the process of overgrowth takes place; the change in staining reaction is difficult to explain otherwise. The formation of cilia follows very soon after the process of overgrowth; thus it cannot be doubted that the impregnation of the ectoplasmic spherules with chromatin stimulates their latent potencies.

Sections stained in thionin and orange G show that the dispersion of the chromatin is by no means confined to the ectoplasm; but that the endoplasm as well is thoroughly interpenetrated with particles of chromatin, which are beyond all question microsomes. The same thing may also be seen in the iron hæmatoxylin preparations, but it is not shown in so striking a manner as by the thionin and orange stain. The bright orange spherules contrast beautifully with the brilliant blue microsomes clustered around them and in all the interspaces. With thionin and orange staining the ectoplasmic spherules continue to take the orange stain even after the intermixture with chromatin but they take the blue also to a certain extent, so that their general effect is olivaceous.

We have seen that in some cases the nucleus draws the ectoplasm into the interior of the egg, so that it is completely surrounded by the endoplasm. It is probable that this is the reason why many eggs fail to reach the stage of differentiation of cilia. Thus in Experiment 9, 1901, very few of the eggs became ciliated; but the preparations show that, in practically all, the nucleus grew and the ectoplasm segregated in the usual fashion. But the sections also show that the internal migration of the ectoplasm is very characteristic of the series.

5. Formation of Cilia and Other Cell Constituents.—The formation of cilia follows hard on the heels of the ectoplasmic overflow. Some uninucleated eggs begin to revolve eight or nine hours after the beginning of the experiment, and others not until much later. The majority, in the case of the potassium series, never reach this stage at all, probably, as suggested before, owing to failure in the process of overflow.

Typically the entire ectoplasmic surface becomes ciliated, but the cultures show great variety of conditions owing to the following circumstances: (1) A good many eggs break into two or more

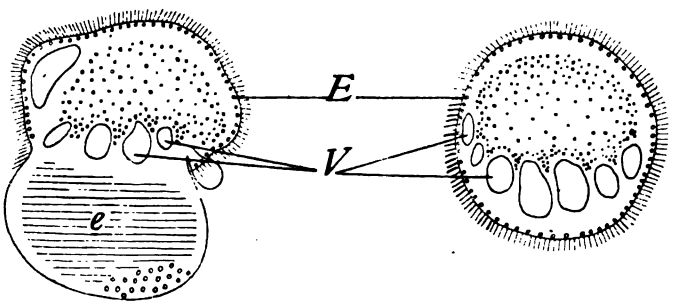

FIG. 69 FIG. 70

Fig. 69. From the same culture as 68; about same age.

Fig. 70. From same culture as 68; about same age. The endoplasm of the egg has been constricted off.

parts owing to excessive development of constrictions between segregated substances (compare Fig. 69). Thus are produced some non-nucleated parts, some parts nucleated and without ectoplasm, other parts nucleated and with relatively little endoplasm. (2) Fusions are very common (see Lillie '02). (3) A larger or smaller part of the endoplasm may remain exposed (compare Figs. 68 and 69).

It is, therefore, not surprising that good examples of clear regional homology with the entire trochophore are rare. Some are, however, found. (The regional homology may be very precise in multinucleated unsegmented eggs; see next section.) The best example of it in uninucleated eggs is shown in Fig. 1 of my 1902 paper. (See also Figs, 68, 69 and 70 of the present

paper.). The reason for the existence of a regional homology is so clear that discussion is hardly necessary. Fig. 70 shows a ciliated part corresponding only to the upper halves of Figs. 68 and 69 and resembling only the exumbrella region of the trochophore.

In general the following statements may be made concerning the differentiation of the uninucleated eggs. (1) Organs are never formed, but only such structural elements as may occur in single cells of the trochophore. (2) Organs may, however, be simulated by the aggregation of the characteristic matter of an organ, for instance in the case of the yellow endoplasm, which simulates the gut of the trochophore, or the row of large vacuoles situated near the upper margin of the yellow endoplasm (*e. g.*, Fig. 69) which simulates the row of vacuoles of the prototroch. (3) The structural elements appear in the same order of time as in the trochophore. (4) The distribution of structural elements tends to resemble that of the trochophore. (5) The yellow endoplasm (yolk ?) is used up, apparently for the maintenance of metabolism, in the ciliated unsegmented eggs precisely as in the larva (see Fig. 71). (6) The apical flagellum is never formed.

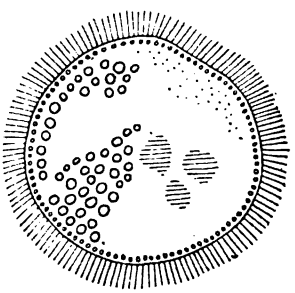

Fig. 71. Ciliated unsegmented egg about twenty-eight hours old. Most of the endoplasm has been consumed. Living.

Inasmuch as a number of conditions were figured and described in my earlier paper (Lillie '02) and because multiplication of examples would be unprofitable, I shall content myself with illustrating in a very few cases the general statements just made.

The first and second statements really require no further elaboration; Figs. 68–71 sufficiently illustrate them.

The order of appearance of the structural elements is: (*a*) cilia; (*b*) intermediate vacuoles; (*c*) ectoplasmic vacuoles. I was much interested in attempting to discover the mode of origin of the cilia. From the fact that their distribution, both in the normal larva and unsegmented eggs, is the same as that of the ectoplas-

mic spherules, I was at first inclined to believe that each spherule was the germ of a cilium, and expected, therefore, to find the same number of cilia and spherules. *The number of cilia seems, however, to exceed the number of spherules, and they do not arise directly from the spherules but from the microsomes between or on the surface of the spherules.* This can be seen very clearly by focusing on the surface of an egg that is acquiring cilia with a 2 mm. oil immersion lens. But there is a very intimate relation, and one can convince himself after the rate has slackened that the spherules vibrate in unison with the beat of the cilia.

The intermediate vacuoles appear between the ectoplasm and the endoplasm soon after the formation of cilia. They are practically confined to the original ectoplasmic hemisphere, and are specially well developed usually just above the margin of the endoplasm, thus in a position corresponding to the prototroch. They appear to correspond to similar large vacuoles situated internal to the nucleus in the cells of the exumbrella and particularly of the prototroch in the larva. But, as they are confined by cell-walls, they tend to fuse and thus to produce a fluid-filled space separating ectoplasm and endoplasm (see figures).

The ectoplasmic vacuoles are much smaller and more refringent, and do not appear until about twenty-four hours from the beginning of the experiment (Fig. 71). They are usually massed in a particular region, corresponding to their distribution in the trochophore where they are very much more abundant in the exumbrella. In the ciliated ova of this age, the yellow endoplasm is much reduced in amount, and the eggs as a rule do not live much longer.

(6). It is a curious fact, observed also by Scott in his observations on Amphitrite, that the apical flagella are never found in these ciliated eggs. This indicates a combination of factors in the normal development that is seldom or never realized under the conditions of the experiments. The explanation is probably as follows: The apical flagella arise at the animal pole, and in Chætopterus one can frequently see one polar body at least at their base. This is, however, the place where the endoplasm comes to the surface; *the apical flagella are therefore endoplasmic*

in their origin. In the overflow of the ectoplasm in the unsegmented eggs this exposure of the endoplasm is seldom, or never, preserved. Thus the formation of the apical flagella would be prevented.

The study of the cytological phenomena in sections has been carried only as far as the overflow of the ectoplasm (about eight hours). Up to this time it is possible to follow the history of the original substances of the egg consecutively. I have not studied the later cytological details; the changes that take place in the structure of the cytoplasm are very profound and complex.

b. Multinucleated Unsegmented Eggs

These are characteristic of cultures of fertilized eggs so treated as to inhibit the process of cleavage or to induce fusion of blastomeres. There is a strong tendency towards polyspermy, if eggs are allowed to stand for several hours in sea-water before being fertilized. Such eggs may break up at the first division into a considerable number of blastomeres, and, in many such cases, the blastomeres soon fuse together, and cleavage planes do not again appear. The protoplasm of the egg of Chætopterus is extremely susceptible to external conditions, and one of the most common evidences of this is the fusion of blastomeres.

In Experiment 7.B., 1904, for instance, the eggs were taken from the female at 9 A. M., and were allowed to stand in sea-water until 11.42, when they were fertilized; a series of eggs was preserved and from them entire mounts and sections were prepared. In the entire mounts of eggs preserved twelve minutes after the addition of the sperm it can readily be seen that there are from three to twelve or more sperm nuclei in each egg. These were also seen in the living eggs under pressure. The polar bodies were formed normally in about twenty-five minutes. Figs. 72 to 75 show the form changes of a single egg, from 12.32 to 12.47 P. M. At first this egg became so lobulated that it seemed as though it were dividing into about ten cells at once; then these lobes gradually disappeared and the eggs returned to their spherical condition. At 12.54 the great majority of the eggs had returned to the spher-

ical condition; about 5 per cent appeared like normal two-celled stages; these were probably monospermic.

In Experiment 7.A. some of the same eggs were fertilized at 10.35 A. M., having remained in the sea-water one hour and thirty-five minutes before fertilization. About 60 per cent of these segmented fairly normally, the blastomeres fused together in the remainder "Intermediate conditions were rare or absent." (Quoted from notes.)

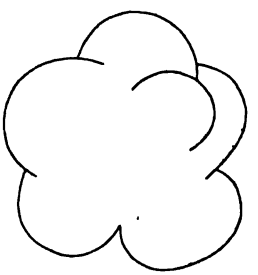

FIG. 72

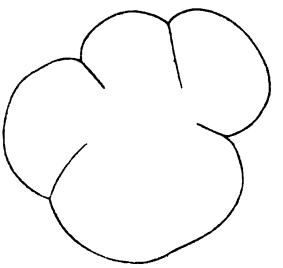

FIG. 73

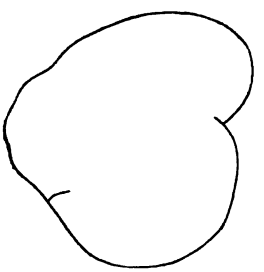

FIG. 74

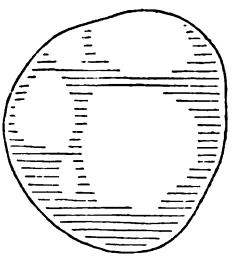

FIG. 75

Figs. 72 to 75. Four views of the same polyspermic egg. The eggs were put in sea-water at 9 A. M. and allowed to stand until 11.42 A. M. when they were fertilized. Fig. 72 was drawn at 12.32 P. M.; Fig. 73 at 12.37; Fig. 74 at 12.44; Fig. 75 at 12.47. At 12.50 the egg was perfectly spherical again and showed no sign of cleavage.

I expect to make a special study of the polyspermy, for which I have the preparations. So in this place I shall consider only a few points. In the polyspermic eggs multipolar spindles arise, and a very uneven distribution of the chromatin results. This is followed by the formation of nuclei of very unequal size, and in the following divisions the mitoses are irregular. Thus there

is an enormous increase of the chromatin, and soon the most of the nuclei cease to be separate in the resting condition, and form a vast reticulum situated between the ectoplasm and endoplasm in the upper half or two-thirds of the egg (Fig. 76). A few small nuclei may remain separate. In the ensuing periods of division the individual karyokinetic figures cannot be observed, and there is a veritable riot of centrosomes, chromosomes, spindle fibers and astral radiations.

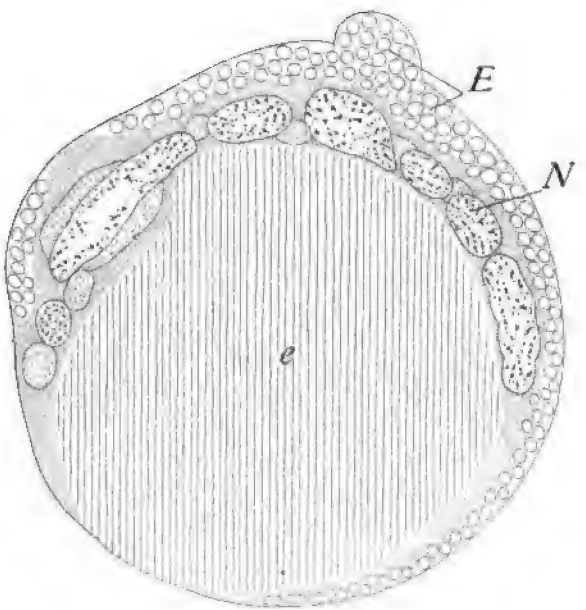

Fig. 76. Optical section of whole mount; multinucleated unsegmented egg, preserved five and one-half hours after fertilization. The nuclei formed an extensive reticulum; the apparently separate nuclei of the figure are the optical sections of the broad strands of the nuclear reticulum.

During this period the ectoplasm becomes very thick at the animal pole, and the endoplasm forms a ball that is attached to the surface at the vegetative pole by the substance of the polar lobe (polar ectoplasm) (Figs. 77 and 78).

Thus it will be seen that the presence of numerous nuclei or of an extended nuclear reticulum, situated between ectoplasm and endoplasm, results in a different arrangement of the ectoplasm and endo-

plasm from that found in the uninucleated ova where the nucleus is central in position. In the multinucleated ova the polar distribution of substances of the ovum does not differ essentially from the normal. It appears to follow that the curious modes of aggregation of the ectoplasm and the endoplasm in the uninucleated ova are due, in the first place, to the lack of the restraining influence of numerous nuclei, and, in the second place, to the fact that, when nuclear influence is established by the enlargement of the nucleus, it proceeds from a single center in place of many.

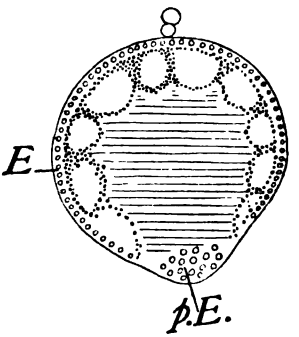

FIG. 77

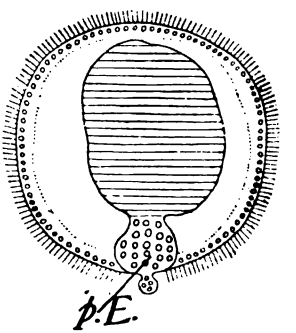

FIG. 78

Fig. 77. Multinucleated unsegmented egg drawn from life four hours and ten minutes after fertilization. The eggs had been allowed to stand in sea-water one hour and fifty minutes before fertilizing.

Fig. 78. Multinucleated unsegmented egg drawn from life eight hours after fertilization. From a culture in which the eggs had been centrifuged one and one-half hours after fertilization.

The maintenance of the normal distribution of ectoplasm and endoplasm in the unsegmented multinucleated ova results in a very perfect homology of the ciliated unsegmented ova with the trochophore. A few examples may illustrate this. Fig. 77 is from a living egg, six hours old. The polar globules show the position of the animal pole; the ectoplasm, endoplasm and nuclei (appearing almost like vacuoles in the living egg) have the positions described for Fig. 76. At the pole of the egg nearly opposite to the polar globules is seen a mass of spherular protoplasm, that corresponds exactly in position and appearance to the mesoblast cells in the normal embryos, which can very readily be seen in the living condition. Thus we can readily homologize ectoplasm,

endoplasm and mesoblast substance with those of the normal larva. Moreover, they have the same positions with relation to one another and to the polarity of the egg as in the normal embryo. The main difference is that the absence of cell-walls permits all of the endoplasm to aggregate in a single mass; whereas in the normal larva it is divided between the cells of the ectoderm, entoderm and mesoderm, though occupying in each kind of cell a position next the segmentation cavity.

In some cases the substance of the polar lobe is partly separated from the yolk-mass proper (Fig. 78). This affords a fine demonstration of the existence of such a substance at the lower end of the yolk-mass. The presence of this substance is perfectly characteristic. It is very adhesive, as union by it of two or three ova is very common.

An interesting addition to the information concerning such unsegmented ciliated eggs is given by staining intra vitam with neutral red. It is then found that there is an accumulation of red granules in the upper hemisphere that correspond precisely in position and staining reaction with those of the trochophore (see Part IV, *d*). The only difference is, that in the unsegmented egg they are more massed, a condition naturally resulting from the absence of cell-walls.

For completeness of demonstration of the principle of germinal localization these differentiated, and yet wholly unsegmented, ova leave little to be desired.

c. Literature and Discussion

Differentiation of entirely unsegmented eggs has been observed in two other genera of polychæta, in Podarke by Treadwell ('02) and in Amphitrite by Scott ('03 and '06). Bullot ('04) failed to observe it in Ophelia, though he looked for it, and he therefore cast some doubt upon its occurrence. I would only point out that the occurrence of segmented ciliated embryos in his cultures by no means demonstrates the absence of the phenomenon of differentiation without segmentation; for Treadwell, Scott and I have all observed the occurrence of ciliated ova both segmented and unsegmented in the same culture-dish. Bullot's figures show

that, in the unfertilized cultures of Ophelia eggs, segmentation was seldom or never, even approximately, normal.

It is clear from the descriptions of these three authors that the occurrence of a certain amount of segmentation is more common under the conditions of the experiment in the forms that they described than it is in Chætopterus, which seems to be peculiarly favorable for the observation of differentiation without any cleavage.

To this list of observations may be added, in all probability, those of Bastian ('05) on a peculiar form of differentiation in Rotifer eggs. Indeed, unless one is willing to adopt Bastian's explanation that particular species of ciliates may arise by direct metamorphosis of rotifer eggs, no other explanation is possible than that he has observed cases of differentiation without cleavage. His peculiar interpretation so colors the description of the observations, that it is difficult to utilize them. But it is probable from the figures that in some cases the eggs form cilia without any cleavage, and that, in other cases, the eggs segment or break into several smaller parts, each of which then differentiates farther without cleavage.

Scott summarizes his results on the differentiation of unfertilized eggs of Amphitrite as follows: "Under the conditions of the experiments certain forms of development occur with or without cleavage and with or without the formation of polar bodies in the unfertilized egg of Amphitrite. Such development takes the form of nuclear divisions, the early differentiation of a layer of ectoplasm, the growth of cilia, the appearance of vacuoles that are found in the fertilized egg of the same age, the development of a brownish pigment, the ameboid movements of the cytoplasm that are connected with cleavage, the ameboid movements at a later stage of development that appear entirely independent of cleavage, and the change in shape of the egg, which in most cases at least is connected with incomplete, arrested or abortive division of cytoplasm. The apical tuft of cilia which is characteristic of trochophores from fertilized eggs is always absent."

Scott justly emphasizes in his theoretical considerations "the intimate relation that exists between cytoplasmic and nuclear

differentiation; the correlation in development between these two factors is very complete where a normal organism results. Inasmuch as the cessation of development is a cumulative process, that is the abnormalities appear in successive transformations of the asters and nucleus, we must look upon the cessation of development as due to incomplete reactions between nucleus and cytoplasm."

Treadwell ('02) observed that ciliated embryos might arise in Podarke without cleavage, and that the differentiation might be carried very far in such cases. One case that he describes is particularly interesting (Fig. 12 of Treadwell); two eggs are fused together and are entirely unsegmented. "Around one portion of the fused mass is a ring of cilia, occupying very much the position, with respect to the cell, of the prototroch in its relation to the trochophore. Not only are the cilia present, but around the embryo, underneath the ciliated band, is an area free from the ordinary pigment of the rest of the cell, but containing granules of a faint yellow color, agreeing in this respect exactly with the prototroch band of the normal trochophore. In this embryo there is, then, not merely a differentiation—without cleavage—of cilia, but of the characteristic protoplasm accompanying these cilia. Or, in other words, we have here in the unsegmented embryo, not merely a differentiation of cilia, but a differentiation of *prototroch cilia*."

The possibility of a considerable amount of embryonal differentiation without either nuclear or cytoplasmic division may be considered established. This in itself is a fact of considerable importance, for it disposes effectually of all theories of development that make the process of cell-division the primary factor of embryonal differentiation, whether in the form of Weismann's qualitative nuclear division, or of Hertwig's cellular interaction theory. Further, the phenomenon establishes firmly, as I pointed out in 1901, the view that the rôle of cell-division in development is primarily a process of localization. This view is a fundamental part of the doctrine of formative stuffs and is held as such by Wilson, Conklin and others. It may of course be variously elaborated, according to whether it is held, for instance, that cell-

walls are impermeable partitions, as Conklin believes ('05, p. 163), or may permit passage of materials through (*e. g.*, by perforation) as Vejdovsky appears to have observed. The meaning that is given to the term "cell-division" would be important in elaborating the theory; for myself I had meant to include in it only the processes of division of the chromosomes, separation of daughter nuclei and the origin of the cell-wall between the daughter nuclei; movements of the substances within the cell, for instance, would be excluded. It seems to me, however, that the elaboration of a theory concerning the rôle of cell-division in development has to meet with considerable difficulties. I would agree, in general with Conklin, that the localization pattern is something more fundamental and constant than the cleavage pattern; yet the fixity of the latter in all ova of the determinate type of cleavage is a marvelous thing, and cannot be fully explained by any mosaic principle (see General Discussion, p. 254).

V. GENERAL DISCUSSION

Embryological and cytological study has advanced beyond the stage of opinion represented by current theories of development. It is generally agreed that the complex phenomena cannot be reduced to the operation of any single factor, and many possible factors have already been more or less thoroughly discussed, but there is no agreement as to the relative importance of the various accepted factors, nor as to their relations to one another in the various embryonic processes.

1. Relation of Nucleus and Cytoplasm

There can be no doubt that the fundamental morphological composition of the protoplasm of the egg of Chætopterus corresponds to the terms of a granule theory and not to those of a filar, reticular or emulsion theory. That a threadlike, reticular or alveolar formation may arise in such protoplasm is unquestionable, but such conditions are secondary ones, due to particular modes of aggregation of the elementary constituents. It is not necessary in adopting a granule theory to go as far as deVries and Altmann

and assume that the granules (pangens of deVries) are the only living elements in the cell, like the bacteria in a zoöglœa. The microsomes appear to be the primary living elements of the cytoplasm; I would not venture to assert that they are the only living elements.[1]

Similarly there can be little doubt that the larger granules, the spherules, are products of the microsomes. The origin of some of these from aggregations or growth of microsome-like bodies could be plainly traced by intra vitam staining; moreover, the spherules follow the microsomes in time of origin in the ovogenesis.

Again there can be no doubt that a very large proportion, at least, of the microsomes are of nuclear origin. A great many occur in the residual substance of the germinal vesicle, and, in the history of the uninucleated unsegmented eggs, swarms of microsomes can be seen to proceed from disintegrating chromosomes just prior to the period of differentiation.

Thus my conclusions correspond to certain of the terms of deVries' theory of intracellular pangenesis (1889), provided that we identify the microsomes with deVries' pangens. This theory involves the following assumptions:

1 That the entire living protoplasm is composed of pangens, which constitute the only living element in it. The pangens are invisibly small.

Now the microsomes are not invisibly small, and I regard them only as the primary living elements; not as the sole living elements.

2. There are as many different kinds of pangens as there are independently variable characters, or independent "factors" composing the complex of the characters of the species.

The only cytological evidences that the microsomes are of different kinds are (*a*) that they produce different kinds of spherules, and (*b*) that they proceed from nine different sources in Chætopterus, viz: the nine chromosomes of the egg-nucleus.

3. All the various kinds of pangens of a species occur in the nucleus, and those existing in the cytoplasm come from the nucleus.

[1] Indeed a large part of the discussion as to what elements of the cell are living and what are not living seems to me to be purely academic, and likely to remain so, until we possess much more satisfactory knowledge of the mechanics of the vital processes.

This agrees with my opinion concerning the microsomes of Chætopterus.

4. The cytoplasm contains essentially only those kinds of pangens that enter into activity in it. Thus in each variety of cell the immense majority of the pangens remain inactive in the nucleus, and only those leave the nucleus and enter into activity in the cytoplasm that represent the specific cell characters to be expressed.

On the cytological side there is no evidence to correspond to this idea. On the contrary all the chromosomes appear to be active in each cell.

5. Pangens multiply both in the nucleus and also in the cytoplasm.

This is certainly the case as regards microsomes in the nucleus, and possibly also in the cytoplasm.

The theory of intra-cellular pangenesis has anticipated certain observations that may be made concerning the elementary phenomena of development. But it is defective in two important respects: (1) it assumes a degree of original diversity, and certain modes of behavior of the pangens (such as the inactivity of the vast majority in each species of cell) that find no justification in our cytological knowledge, but only in alleged theoretical necessities; (2) it provides no explanation of an essential part of embryonic development, viz: the spatial arrangement of organs and their sequence in time, in short, the unity of the organism by which alone is the possibility of self-sustenance guaranteed. Weismann's theory of germ-plasm proceeds yet farther in unwarranted assumptions as to the original complexity of the germ-plasm, but includes an explanation of the spatial arrangement of organs and their sequence in time, by providing for these factors in the architecture of the germ-plasm.

2. *The Original Diversity of Organization*

All theories postulate a certain original diversity or complexity of organization as the starting point in embryonic development. Now we must inquire what we mean by *original?* For some the fertilized ovum constitutes the starting point; but it is clear, on consideration, that it is relatively far removed from the actual

origin. Only that can be *original* which is continuous through the series of generations, viz: the germ-plasm in Weismann's terminology, which we identify with the chromosome group. The entire history of the ovogenesis, as Wilson has repeatedly pointed out, forms part of the embryonic development. The original diversity of organization is, therefore, contained in the specific chromosome group, which observation has shown to be transmitted from generation to generation.

The next question is as to the degree of original diversity within this chromosome complex. I would maintain that the estimation of this should rest primarily on the observable facts and not on a mental projection to the lower plane of germ-plasm of the complexity of the higher plane of the adult organization. The visible diversity of the chromosome complex is usually only quantitative, that is, it consists of a definite number of parts, the individual chromosomes. In some cases qualitative differences are also observable, for the individual chromosomes may differ in size and behavior (Montgomery '01, Sutton '02, Wilson '05); Boveri ('02) has shown also for the echinids, by some ingenious experiments that, though all the chromosomes are alike morphologically, the individual chromosomes are probably non-equivalent physiologically. There is, therefore, good reason for believing that there are at least as many different kinds of original substances in the germ-plasm as there are chromosomes. Considerations as to valency of chromosomes may, perhaps in the future, tend to equate differences in the number of chromosomes in different species; recent observations of McClung ('05) have furnished some arguments along this line. To maintain that there are no more original germinal substances than there are actual unit chromosomes may, perhaps, be too extreme a position; but it seems to me sounder by far and likely to prove more fruitful as a working hypothesis than the assumption that the germ-plasm is a microcosm of "determinants" of all the characters of the species.

I do not believe that any considerations as to the potencies of the germ-plasm are valid as arguments for the amount of the original diversity, because, if the validity of such arguments be

recognized, there remains no standard but the arbitrary judgment of the individual. Apart from the *a priori* difficulty of accounting for the phenomena of heredity there is no reason for assuming the existence of a large number of original germinal qualities. But, seeing that any species is as distinct from other species in the stage of germ-plasm as in the adult condition, the original germinal qualities, whatever they may be, must bear the stamp of the species.

At present we have no accurate means of estimating the degree or nature of the differences between the chromosomes, but I believe that certain statements may be made about them that follow logically from our present knowledge. In the first place, the differences cannot correspond to the differences between organs or regions, either of the embryo or adult, because the doctrine of the individuality of the chromosomes teaches that each cell receives a descendent of each chromosome. The whole economy of nature forbids us to believe that each cell possesses arm, leg, brain, liver, lung, etc., chromosomes, of which only one class enters into activity in any given tissue, the remainder lying idle. *The fact of the uniform distribution of all chromosomes to all tissues proves conclusively, either that all chromosomes are alike, or that each represents some character of the entire organism. As we have accepted the view that they are originally unlike, we must adopt the second alternative.*

The only observations that we have connecting a particular chromosome with a particular set of characters are those of McClung ('02) and Wilson ('05), according to which the accessory or idiochromosome is a sex-determinant. Now sex is preëminently a character of the entire organism. My hypothesis is, that each chromosome represents some such character or group of characters. It is difficult to imagine what such characters may be; we need a new morphology for their enumeration, and it is to be hoped that this will come from the breeders' experiments; for the only clue that we have to the relation between chromosome-characters and species-characters, consists in the parallelism between the reduction-phenomena in the germ-cells and the Mendelian ratios in inheritance. We might hope, therefore, to get at the nature of chromosome-characters by an enumeration

of the various kinds of characters that are inherited in Mendelian proportions. To attempt this in detail would be too great a task, but they include such characters as color (*e. g.*, inheritance of albinism, or green and yellow endosperm of peas, etc.), stature, pubescence, etc., that are not special characters of particular organs but of the whole organism.

We must be careful, however, to avoid a pitfall here in assuming that there is any *resemblance* between species-characters and chromosome-characters. There is at most only *correspondence* due to genetic connection; and any imaginable degree of knowledge of the unit species-characters would not furnsh a particle of information as to the nature of the original unit germinal characters.

3. *Properties of the Whole (Principle of Unity)*

If the first step in any theory of embryonic development must be certain postulates concerning the original diversity of organization, the second step must be an explanation of the physiological unity of the organism in all stages of its development; for the two main facts concerning any organism are that its parts are diverse, and that it is, nevertheless, a physiological unit. The traditional view, held by many embryologists at the present day, is that the physiological unity arises in the course of embryonic development by the secondary adaptation of originally independent parts to one another. But this explanation has, in my opinion, become untenable, and must be replaced by the view that *there are certain properties of the whole, constituting a principle of unity of organization, that are part of the original inheritance, and thus continuous through the cycles of the generations, and do not arise anew in each* (compare Lillie, '01, p. 275). Weismann places this principle of unity of organization in the architecture of the germ-plasm, but, as I cannot accept his view of vast complexity of the germ-plasm, neither can I accept this principle in the sense of Weismann. My own views agree most nearly with those expressed by Whitman in his paper on the Inadequacy of the Cellular Theory of Development ('93). In this paper

Whitman uses the term organization to express what I have termed above, *properties of the whole or principle of unity.*

If any radical conclusion from the immense amount of investigation of the elementary phenomena of development be justified, this is: that the cells are subordinate to the organism, which produces them, and makes them large or small, of a slow or rapid rate of division, causes them to divide, now in this direction, now in that, and in all respects so disposes them that the latent being comes to full expression. We see this in the adaptiveness of the process of cleavage of the ovum (Lillie '95, '99; Conklin '96–'97; Meisenhemier '99), in the regeneration of a starving planarian constantly suffering a diminution in the number of its cells while its structure is increasing in complexity (Lillie '00, Schultz '04), in "regulation," and in all cases of morphallaxis (Morgan '00), whether in a protozoan or a metazoan. The organism is primary, not secondary; it is an individual, not by virtue of the coöperation of countless lesser individualities, but an individual that produces these lesser individualities on which its full expression depends. The persistence of organization is a primary law of embryonic development.

I believe that this conclusion is strongly reinforced by my observations on differentiation without cleavage; for here we see the various substances of the ovum marshalled in order, disposed in a bilateral arrangement and fashioned in the form of a larva; and we see the cilia and other cell-constituents arise in the appropriate locations—and all this without the need of even a single nuclear division. The question arises whether these phenomena could not be explained by assuming appropriate attractions and repulsions between the elements of the different classes of substances, the spherules and microsomes. Although such attractions and repulsions undoubtedly exist, and although they appear to me to constitute an important elementary morphogenic factor, yet I find the assumption inadequate to explain the orderly arrangement of the processes collectively. It seemed at first that the polarity of the ovum might be explained by assuming that the arrangement of endoplasmic substances was produced by mutual attractions and repulsions; but it was found that no alteration of

the arrangement of the endoplasmic substances modified the direction of polarity. Similarly the bilateral polarization of the first cleavage spindle and many aspects of the later cleavages appear to be independent of any chance arrangement of endoplasmic spherules.

Thus there is an apparent inversion in the sequence of embryonic phenomena, by virtue of which those characters that we would expect to appear late, such as the general form and proportions of the embryo, manifest themselves first, and thus lend to the subsequent phenomena an adaptive aspect; as though that which was to be explained preceded the phenomena that could alone account for it. *It is obvious, however, that the adaptiveness of development does not constitute an explanation, but is, on the contrary, itself one of the chief phenomena to be explained.*

The principle of unity transcends all forms of visible diversity hitherto observed; it is a property of the whole distinct from the discernible properties of the parts. Undoubtedly it is capable of further analysis, and it must ultimately be derived from particular relations and properties of material particles. In embryonic development it reveals itself first by axial polarization, second by bilateral polarization and determination of the localization pattern, third by adaptation in cleavage, etc. Analysis of some of these phenomena may some day give a clue to this most mysterious of embryological phenomena.

Morgan ('04) has attempted an "Analysis of the Phenomena of Organic 'Polarity,'" based on the phenomena of regeneration. He concludes that "by means of three assumptions—of totipotence, of heterotropy and of organization-power—we can explain the main features in the result. Each assumption is, moreover, a direct deduction from an experiment or observation." In a footnote he adds, that "the same explanation applies to the development of the egg." By "heterotropy" he means that "the material," though totipotent "is somewhat different at every level, and that this difference corresponds *in kind* to the character of the body at each level." The "organization power," as I understand it, is the same as the "centripetal influence" which, "acting from the surface inward, determines the organization of

the new parts. The action of this centripetal influence is on the new parts as a whole, and determines the relative location of each organ." Later on he suggests that "for want of a better term, we may provisionally call the property of living material to assume a specific form, the property of *formative organization.*"

It is clear that Morgan here offers an analysis of much more than is generally included under the term polarity; indeed he offers an analysis of regeneration and embryonic development as a whole. It is interesting to note that he defines the factor of "formative organization" so as to be similar to what Whitman calls organization simply, and what I have called influence of the whole. It would be essentially the same idea, if it included heterotropy also, and the latter appears to me to be subordinate to the former principle.

4. *The Mosaic Theory of Development*

The mosaic theory of development of Roux, excepting that inessential part concerning qualitative nuclear analysis, has been strongly supported by the facts of cell-lineage and the recent work of Boveri ('01a), Fischel ('03), Wilson ('04a and b), Conklin ('05a and b), Zeleny ('05), Yatsu ('04) and others. Wilson, Conklin and Fischel especially have shown conclusively that in mollusca, ascidians and ctenophores, the mosaic character of development is based on the principle of germinal localization originally enunciated by His. The work of these authors is too recent and well known to need review here; I only wish to say that I fully accept the results, and am prepared to abide by the theoretical necessities resulting from the assumption that the cleavage mosaic in Chætopterus is as definite a mosaic of potencies as it is in Patella, where each cell of the cleavage-mosaic up to the thirty-two celled stage, or later, differentiates after isolation in substantially the same manner as when forming part of the whole.

But Wilson ('04b) thinks that this result is inconsistent with the conclusion stated by me in 1901, "that the entire organism in every stage of its development exercises a formative influence on all of its parts," although he does not doubt "that this position,

with proper qualifications, is well grounded." He then goes on to say that "it is clear that the primary localization of formative stuffs in the unsegmented egg is essentially an act of the "organism as a whole;" and even though a complete preformation and prelocalization of specific stuffs for every cell and tissue were assumed —and I believe with Boveri and Fischel that such an assumption is not necessary or even probable—we should not escape the necessity for assuming such action of the whole." But in the same paragraph, while assenting to Whitman's saying that "organization precedes cell-formation and regulates it," he takes issue with him on the ground "that the cytoplasmic aggregation or "organization" is a progressive or epigenetic process."

As I understand Whitman, "organization" is what I have called "action of the organism as a whole;" Wilson has either understood the matter differently, or has forgotten this, when he identifies "cytoplasmic segregation" and "organization." Organization, or action of the organism as a whole, is something that precedes and regulates cytoplasmic localization as much as it does cell-formation. If this fact were clearly kept in mind I think that Wilson would find less difference between Whitman's views (and mine) and his own, than he suspects; for the same mistaken identification of organization with cytoplasmic localization reappears in the succeeding remarks.

But I do not mean to assert that my views are identical with those of Wilson even when this allowance is made, for he seems to believe that the action of the organism as a whole ceases when once the localization pattern is determined, and that thereafter it is a question of self-differentiation of independently developing parts with a certain amount of correlative interaction[1] of cells.

[1] It seems necessary to make a special statement of the opinion that I hold concerning the distinction between "action of the organism as a whole" and the principle of correlative differentiation. By the former I mean, to use the words of my paper on the organization of the egg of Unio, "that the entire organism in every stage of development exercises a formative influence on all of its parts." The principle of correlative differentiation, as I understand it, involves all actions of the *intraorganic environment*, as I expressed it in my "Experimental Studies on the Development of the Organ in the Embryo of the Fowl" (1903), that is, "that the rate, degree or mode of differentation of any embryonic rudiment is dependent on some part or parts of the same organism external to itself." It will be seen that, as thus conceived, there is an important difference between the two principles, and that the principle of correlative differentiation would not include the principle of action of the organism as a whole without a considerable extension of its usual meaning, which seems to me undesirable and likely to be confusing.

It appears to me on the contrary, *that the action of the organism as a whole is a continuous process; that physiological unity exists in every stage, and not merely sporadically; no matter to what extent the mosaic principle may apply.* We have to deal with diversity in unity, and unity in the midst of diversity as the two fundamental properties of organisms. The phenomena of regeneration and regulation are incomprehensible on the basis of a *pure* mosaic theory of development. Wilson does not fail to recognize this principle here and there, as in cases of regulation, but it seems to me that he has not given it sufficient weight, and has not kept clearly in mind the distinction between this principle and the derivative condition of germinal localization.

I would like also to give what seems to me the explanation of Wilson's conclusion that "cytoplasmic segregation is a progressive or epigenetic process," based on the results of Boveri, Yatsu, Zeleny and himself. In general it may be said that these authors have found that there is a progressive limitation in the potencies of parts of the egg from the time of the rupture of the germinal vesicle to the eight-celled stage or beyond. Now in Chætopterus there is a visible segregation of substances *already present*, described in Part III, 2, beginning with the breaking of the germinal vesicle, and continuing to the third cleavage at least, by means of which a new germinal topography is produced. Assuming that these substances have limited potencies, as has been demonstrated for similar substances in other eggs, the redistribution of them would inevitably bring about such a result as these authors have described, because the new topography is much more precise than the original one. The rearrangement is an "epigenetic process," if you please; but only the topography is new, not the substances. There is a great deal of evidence in the literature that a similar redistribution is characteristic of the maturation period in most phyla. On the other hand I believe that a true epigenetic process begins with the first cleavage in the production of new substances from the nuclei, and undoubtedly plays a part in the progressive limitation of potency of the blastomeres; but, I believe, only a small part *at first.* (To avoid misunderstanding,

I would state that it is my view that the specific formative stuffs of the later development arise after the process of cleavage has begun.)

Differences in time of origin of formative stuffs no doubt exist in different kinds of eggs, but I do not believe that the differences are so extreme as some would appear to think. Determinateness of cleavage may be a measure of the extent and precision of their localization prior to cleavage, but I think it must be a very *inexact* measure. I fully agree with Whitman that "cell-orientation may enable us to infer organization, but to regard it as a measure of organization is a serious error;" however, one must keep in mind that "organization" precedes and controls "localization," and not confuse the two terms. When this is done I can also agree with Wilson that "a highly differentiated initial cleavage pattern is, therefore, *ipso facto* evidence" (in some degree), "of a high degree of initial cytoplasmic *localization*" (last italics mine).

5. *Concerning Formative Stuffs*

Much has been written of late concerning formative stuffs.[1] (Morphoplasmic stuffs of Wilson, organ-forming substances of various authors.) Unfortunately their actual physical characteristics have been but little examined; indeed, in most cases the so-called organ-forming substances are really germinal areas probably including a variety of substances, and distinguishable only by their localization and by the useful but superficial character of color. As I have attempted to show, the specific character of the substances in Chætopterus is given by the spherules. It is certainly reasonable to expect that formative stuffs in other ova may be differentiated by microchemical methods. Previous observations on this point have been more or less incidental, so that generalization would not be profitable.

According to the theory of formative stuffs developed in the preceding pages, a series of stages characterizes each kind before it reaches its definitive histological condition. First, its origin,

[1]These "formative stuffs" do not conform to the older conceptions of Sachs, who postulated circulating fluids of formative function. They are supposed to be varieties of protoplasm that probably do not circulate extensively from cell to cell.

in the form of microsomes, from the nucleus; second, the formation of granules of a different order, from the microsomes, the spherules; third, the addition of new nuclear derivatives. (The third stage is necessary for there is no final stage of differentiation in non-nucleated parts; in the uninucleated unsegmented eggs the third stage is clearly marked morphologically by the diffusion of chromatin particles among the spherules.) Fourth, definitive histogenesis (*the morphogenic reaction*).

The theory of formative stuffs does away with any "determinant" hypothesis. "Characters" are not due to "unfolding" of the "potencies" of "determinants" but are results of morphogenic reactions between two or more formative stuffs. The "character" need no more be preformed in the reagents (formative stuffs) in the case of a morphogenic than in the case of a chemical reaction. But I do not mean to imply that the morphogenic reaction is a simple chemical reaction, nor that, after it has taken place, the character need appear at once in its definitive form. The morphogenic reaction is probably often of the nature of a response to a stimulus, a phenomenon of irritability, and the definitive "character" resulting may come to expression slowly.

6. *Nuclear Specification*

The logical consequences of the preceding conclusions cannot be avoided: it is clear that the nuclei cannot produce the same kinds of formative stuffs in successive stages of ontogenesis, unless we assume that the organism as a whole manufactures different things out of the same substances at different times and in different places. This appears to be so improbable an assumption that there is no escape from the conclusion that *the nuclei undergo progressive differentiation;* in other words, that each successive ontogenetic stage is preceded by a corresponding nuclear phase.

The possible modes of nuclear specification are: (1) that different chromosomes represent successive ontogenetic phases, and that, therefore, in any given phase the majority are inactive. The objections to this view appear to me to be fatal; for, in the first place, there is not a sufficient number of chromosomes to satisfy

the conditions of the hypothesis; and in the second place, there are grave objections to the hypothesis of inactive chromosomes in any stage of development. This is, however, substantially the view that Wilson presents ('05, p. 292). (2) The second possibility would be that all chromosomes are active in the various stages of ontogenesis, but that only a part of each is active at any given stage. In other words, that each chromosome contains the determinants of all stages, as Weismann supposes, and that these enter into activity successively. This would, however, presuppose an enormous amount of original diversity, an idea that we have already specifically rejected, besides being open to the general objection that the major part of each nucleus would necessarily be supposed to be inactive. (3) Both the foregoing are essentially preformation hypotheses. The third possibility is that the postulated progressive development of nuclei is essentially an epigenetic process.

It appears to me that all the well-established physiological and embryological data point toward this conclusion. Yet, from Nägeli down, nearly every writer on the subject of heredity postulates a degree of original preformation in the idioplasm or nucleus, corresponding to the amount of morphogenic activity supposed to be exercised by it in all stages of the life history. According to Nägeli "every perceptible character is represented in the idioplasm by a rudiment" (Abstammungslehre, 1884, p. 23). According to deVries it is necessary to assume original specific pangens for every heritable property (Intracellular Pangenesis, 1889). Weismann postulates a determinant for every independently varying part. The embryologists have usually not entered into this question; but Wilson has gone so far as to state ('05a) "that the germ consists of two elements, one of which undergoes a development that is essentially epigenetic, while the other represents an original controlling and determining element. The first is represented by the protoplasm of the egg. The second is the nucleus, which, as I have attempted to show, must apparently be conceived as a kind of microcosm or original preformation, consisting of elements which correspond, each for each, to particular parts or characters of the future organism."

According to these writers, therefore, all the characters that are ever to be impressed by the nucleus on the cytoplasm are represented by original preformations in the nucleus. Such a conclusion appears to me to be practically a negation of the evidences of our senses. If such a degree of original diversity is really preformed in the chromosome-complex, it is inconceivable that it should not reveal itself to some one of our senses by variety of behavior or reaction. Moreover, there is not room in the known laws of chemical combination for such diversity of substances within the chromatin of a species as these preformation hypotheses require. It seems to me that all *a priori* considerations should be ruled out of court, unless we are willing to transform biology into a branch of metaphysics dealing with potencies and latencies.

If nuclear specification is to be considered an epigenetic process, the causes thereof may be conceived either to be in the environment of the nucleus, viz: the body of the cell, or to lie within the nucleus itself. In the former case we should have to assume that the locations of nuclei in different parts of the original germinal topography may act as stimuli on the nuclei to differentiate them in various directions. The original cause of cytoplasmic diversity has been traced back to the nucleus, but I do not think that it is necessarily illogical to reverse the order and assume that the cytoplasmic diversity may be a cause of new nuclear diversity. On the contrary it is a widespread biological phenomenon that secretions of the organism react on the organism itself in various ways.

On the other hand I cannot avoid the conclusion that the progressive development of nuclei is to a great extent a process of self-differentiation. There are certain unavoidable corollaries of the argument bearing on the question. If descendants of each original chromosome are transmitted to each cell of the organism, and if each chromosome, therefore, represents some character of the entire organism, as we concluded before, it must follow that each has a series of forms of expression suitable to the successive ontogenetic stages. Thus, if chromosome x, for instance, expresses itself by pigmentation in the adult condition, it must have had

some different form of expression in stages prior to the appearance of pigment. There is, in other words, a correspondence between the mode of expression of a chromosome, and the ontogenetic stage reached by the entire organism. *There must, therefore, be a progressive evolution of the chromosomes of the same general character in all cells; but this need not exclude local specialization of the nuclei also.*

The differentiation of any particular cell would therefore be the result of an interaction between a specific formative stuff or stuffs, inherited from previous generations of cells, and a new material derived from the nucleus. Variation in either factor would give a different result. Thus, for instance, all cells of the embryonic epidermis might be supposed to contain similar stuffs originally; their special lines of differentiation would then be dependent on the nature of the final nuclear stuff. If, now, we suppose that this may vary with the different external conditions, the line of differentiation would vary with the latter.

To take a specific instance, we may explain Lewis' ('04) interesting discovery that a lens may arise in tadpoles from any part of the embryonic epidermis that is brought into suitable relations to the optic vesicle, by the hypothesis that the stimulus of the optic vesicle causes a different kind of nuclear secretion in the epidermal cells acted on than in others. In this case the postulated nuclear differentiation would result from the environment. If such a case may be considered typical, we might perhaps generalize by saying that the *progressive evolution* of chromosomes common to all cells is a process of self-differentiation, but that local specifications may result from action of the environment.

According to this conception, therefore, there is an orthogenic and epigenetic progressive development or evolution of the somatic nuclei during the development of the individual, that is common to them all, and in addition local specifications characteristic of particular regions and organs. The latter are subordinate modifications within the limits set by the ontogenic stage reached in the evolution of the somatic nuclei of the individual. There would also be involved in the general conception a theory of continuity of the germ-plasm, similar to Weismann's, viz: that the nuclei of the

"Keimbahn" undergo neither evolution nor specification, except such evolution as may be phylogenic in its character.

Without developing the idea any farther in this place I think it will be seen that the view is not inconsistent with the biogenetic law, and that it may be made part of a larger theory of phylogenic development. It seems to me that some such ideas as these result logically from our present cytological knowledge and, indeed they have arisen in my mind in the attempt to interpret current cytological conceptions. They are presented in no dogmatic spirit, but in the hope of stimulating discussion.

7. *Summary of Discussion*

The main points of the discussion may be summarized thus:

(1) The chromosome group of the species contains the total sum of the material transmitted from one generation to another.

(2) The microsomes arise from the chromosomes and constitute the primary cytoplasmic element. They produce the various formative stuffs.

(3) The original diversity, by which I mean the actual degree of heterogeneity of the chromosome group, is probably relatively slight.

(4) There is an original principle of unity, action of the organism as a whole, which expresses itself by axial and bilateral polarization (thus determining the segregation pattern) by adaptation in cleavage, and probably in various other ways, and which is continuous from generation to generation. Only its mode of expression changes and this in accordance with the stage of development of the organism. The unity of the organism does not arise by the secondary process of division of labor.

(5) Apart from the postulated original diversity and the action of the organism as a whole, the entire development is epigenetic.

(6) Each chromosome probably represents in each stage some property of the entire organism.

(7) Each ontogenic stage is preceded by a corresponding nuclear phase; in other words, nuclear evolution is the primary factor in the determination of embryonic stages.

(8) Nuclei probably also undergo local specification as a result of varying intraorganic environment, and possibly also through action of the organism as a whole.

(9) By virtue of the two modes of nuclear evolution and specification, different kinds of formative stuffs arise at successive phases of the ontogenesis and in different parts of the embryo.

(10) The final histogenesis of any cell depends upon interaction of the formative stuffs already present in the cytoplasm with the last formative stuff derived from the nucleus.

(11) The nuclei of the "Keimbahn" undergo neither evolution nor specification except such as may be of a phylogenic character.

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DESCRIPTION OF PHOTOGRAPHS.

The author is greatly indebted to Misses Catharine Foot and E. C. Strobell for the photographs which were taken and printed by them.

All the photographs are from living eggs. The magnification in all is 237 diameters.

All the eggs shown were centrifuged, about 3000 revolutions in one minute, and (with the exception of A and H) then fertilized. *A* to *I* inclusive, show the early stages of such eggs up to the first cleavage, one and one-quarter hours. J, K, L and M were taken from six and one-quarter to seven hours from the time of fertilization.

The first group illustrates the stratification of the egg substances produced by the centrifuge and its relation to polarity, the axis of the egg standing vertical in each case (except in Photograph A, where the axis is not known). The large, dense area seen in each photograph is the massed spherules of the endoplasm, the smaller is the "gray cap" or residual substance of the germinal vesicle. The clear band is seen between them. The photographs show the ectoplasmic layer very distinctly, especially, by contrast, just external to the endoplasmic mass.

Except for the outlines of the polar bodies in B, D, F, G and I, the photographs have been reproduced without retouching.

A. Living egg, unfertilized, shortly after centrifuging. The polar area cannot be seen, hence the relation of the strata to the polarity is not known.

B and C. Two views of one egg with two polar bodies. Centrifuged 8.45 A. M. Fertilized 9.17 A. M. Photographed forty-eight minutes after fertilization. C is a high focus to show the gray cap, B a lower focus to show the polar bodies. The plane of stratification is nearly at right angles to the axis of the egg.

D. Centrifuged 8.45 A. M. Fertilized 9.17 A. M. Photographed 10.13 A. M. There are two polar bodies. The plane of stratification is inclined about 90° to the axis of the egg.

E. History same as D. Photographed 10.20 A. M. There are four polar bodies, a condition observed only once. Plane of stratification inclined about 45° to the axis of the egg.

F. History similar to D. There are two polar bodies. The polar lobe is beginning to form. Plane of stratification inclined about 120° to the axis of the egg.

G. Same egg as shown in F. Photographed five minutes later.

H. Centrifuged 9.08 A. M. Unfertilized. Photographed 10.45 A. M. The gray cap surrounds the maturation spindle and is therefore ring-shaped. During the time that has elapsed since centrifuging the gray cap has spread out considerably.

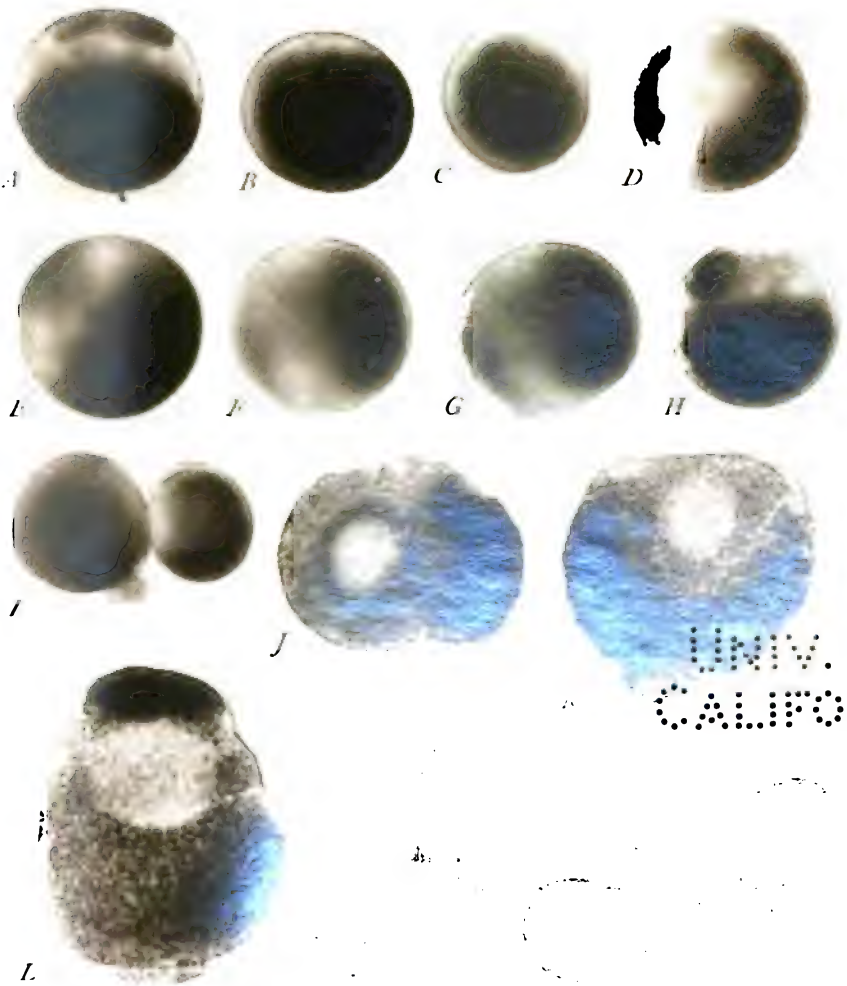
I. History similar to D. Two-celled stage nearly complete. Polar lobe is nearly at its height. Its substance contrasts strongly with the endoplasm. Most of the gray cap is in the smaller cell and the larger is filled almost entirely with endoplasm.

J. Centrifuged 9.08 A. M. Fertilized immediately. Photographed 3.30 P. M., six hours and twenty minutes after fertilization. This is a uninucleated unsegmented egg showing the large clear nucleus and the chromatin halo surrounding it; distorted and flattened by pressure.

K. History same as J. Photographed 4.10 P. M., seven hours after fertilization. The chromatin halo has risen to the surface where it is mingling with the ectoplasm. Egg distorted by pressure.

L. History same as J. Photographed 3.52 P. M., six hours and forty minutes after fertilization. Egg is flattened by pressure. Three-banded condition. The shadow of the ectoplasmic band may be seen on the surface of the nucleus.

M. History same as J. Photographed 3.45 P. M., six hours and thirty-five minutes after fertilization. Three eggs are shown in one field of the microscope, the one to the left is segmented (abnormal) the central one is multinucleated unsegmented; the egg to the right is uninucleated unsegmented. Shown for comparison of the three main conditions found in such cultures.



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THE BIOLOGICAL SIGNIFICANCE AND CONTROL OF SEX[1]

THE BIOLOGICAL SIGNIFICANCE OF SEXUAL DIFFERENTIATION—A ZOOLOGICAL POINT OF VIEW

THE line of descent in multicellular animals is through a continuous or discontinuous series of sexual generations. In the latter case, there is alternation of generations, either of asexual and sexual or of parthenogenetic and sexual generations. So far as I know, the sexual generation is never absent in the first kind of alternation; there are, however, some parthenogenetic species in which males have never been found, though the structure of the females, or the natural history of the race, proves the former existence of males. There is only one feature common to all forms of sexual reproduction, and that is the union of ovum and spermatozoon to form a single cell, which has the capacity of developing into a new individual of the species. The biological significance of sex must, therefore, lie in the process of fertilization; and the interpretation of the fundamental significance of fertilization must be the answer to our problem.

[1] One of five addresses given before the American Society of Naturalists at Columbia University, New York, December 28, 1906.

Now fertilization is a more general phenomenon than sex itself, for it is characteristic of the Protozoa in the form of conjugation; and it appears to be a growing conviction among students of Protozoa that conjugation is universal in this group. Fertilization brings about biparental inheritance or amphimixis, and some have regarded this as its chief function, in view of the great importance of amphimixis for the process of evolution. But most zoologists regard amphimixis as a secondary function of fertilization, and find the chief significance of fertilization in the satisfaction of a periodic physiological need of the organism. The ovum usually requires fertilization as a stimulus to development; without it, in most animals, the processes of development either do not begin or soon cease. Observations on normal and artificial parthenogenesis demonstrate that it is not an indispensable requirement for development; however, in most parthenogenetic species fertilization-need arises in certain generations that alternate more or less regularly with the parthenogenetic ones; and those parthenogenetic species in which males are unknown have descended from sexual species, and moreover belong to specialized groups on one side of the main trend of evolution. Among Protozoa there seems to be a periodic need of fertilization to maintain the capacity of the species for reproduction.

We may then say, with the qualifications already indicated, that among animals at least the law of conjugation is as universal and imperative as the law of hunger. It is thus one of the most general of biological phenomena, with an element of obscurity

in it that does not inhere in any other major problem of biology; for, as katabolism is combustion, the need of hunger to incite the individual to the taking of food is obvious; as the individual survives by adjustment to its environment, it must possess irritability and motility; but why the same food that satisfies for so long fails ultimately to support ebbing vitality among Protozoa, why the line of descent in Metazoa should pass through sexual generations —this is the mystery of physiology; and that salvation of the race should reside in the union of particular cellular individuals, has in it none of the obviousness of the individual's preservation by the taking of food.

The nature of the periodic need for fertilization has been differently conceived by different writers. Weismann believes that fertilization-need has arisen in the course of evolution in order to ensure the advantages of amphimixis to the race; it has no fundamental physiological basis. R. Hertwig propounds the hypothesis that there is an innate tendency to acceleration of the vital processes, due to the gradual adaptation of nucleus and cytoplasm in their copartnership, which proves gradually harmful and ultimately fatal. Fertilization checks the acceleration by introducing a foreign nucleus, unaccustomed to the protoplasmic milieu; but the new partners in the vital process gradually accelerate the speed until a second fertilization again checks the dangerous pace. Fertilization thus marks the return to a state of stability from a state of extreme cellular lability. Herbert Spencer, on the other hand, regards the vital processes as tending

towards a state of equilibrium or fixed stability; fertilization restores the labile condition of the cell. According to Geddes and Thompson, fertilization may be compared to mutual digestion and may have arisen from a nutritive want. "With the differentiation of the elements on anabolic and katabolic lines, the nature of the fertilizing act becomes more definite. * * * The union of the two sets of products restores the normal balance and rhythm of cellular life."

The point of view that has been, perhaps, the most acceptable to zoologists was stated originally by Bütschli, Engelmann, Minot and Maupas, and was based primarily on the study of conjugation in infusoria. It was discovered that, during the series of asexual generations, there is a gradual diminution of vitality expressed in a reduced rate of division and in certain morphological changes that may be collectively designated senescent. Conjugation changes all this; senile processes cease, the division rate is restored. Thus conjugation apparently reverses the process of senescence, causes rejuvenescence. By an extension of this idea it was assumed that in Metazoa the fertilized ovum starts out charged with abundant vitality, which is, however, gradually exhausted, and the race is saved only by fertilization, which is here also interpreted, by a reckless transfer of terms, as rejuvenescence.

The majority of zoologists appear to be agreed that fertilization-need is a primordial physiological condition, more than a mere adaptation to ensure amphimixis; and though there are weighty authorities on the other side, this point of view ap-

pears to me to be right, even though the theories of the nature of the need and its satisfaction are inconsistent. The idea of Spencer is too indefinite to serve either for foundation of a more extensive theory, or as basis for observation and experiment. That of R. Hertwig is in opposition to so many known facts as to be untenable. The conception of rejuvenescence has a flavor of mysticism, and involves a confusion of ideas. It implies that the gametes are senescent before fertilization, but the only significance of the term senescent is in its application to the soma. That the germ-cells before fertilization are old, in the sense that tissue-cells become old, would be asserted by no one. They are, on the contrary, the spring of eternal youth, and all that can be asserted objectively is the necessity of fertilization for their continual functioning.

Even in the case of Protozoa there is no reason for assuming that the part principally concerned in conjugation, the nucleus, is itself old; the cell-body undoubtedly becomes old, and after conjugation it apparently resumes its vigor. But the latter process is more correctly interpreted as renewal instead of rejuvenescence; the old body is sloughed off a little at a time and gradually renewed after conjugation from the germ-plasm, because it can not be cast off an entire dying body as in Metazoa.

I believe that Weismann is correct in his contention that the conception of rejuvenescence has not a shadow of support among the Metazoa, indeed would never have been conceived from what we know about the Metazoa themselves; and also that the con-

ception is baseless as applied to Protozoa. The conception of germ-plasm and soma is as necessary for Protozoa as Metazoa, and the conception of senescence is unmeaning as applied to germ-plasm. But Weismann's conclusion that the Protozoa are potentially immortal does not follow; the protozoan soma is no more immortal than that of the Metazoa, and it is as little subject to rejuvenescence.

No theory of sex can be consistent that divorces the physiological significance from the causes of sex-differentiation. In the physiological significance, that is, in the nature of the fertilization-need, we must find the primary cause of sexual differentiation. Richard Hertwig has been one of the very few to recognize this axiomatic principle; but he nevertheless states two hypotheses, one of the physiological significance of fertilization, the goal and ultimate attainment of sex-differentiation, and the other of the causes of sex-differentiation itself, and these have no logical connection. The main value of Geddes's and Thompson's otherwise vague and unsatisfactory theory of sex lies in their appreciation of the connection between the physiological significance and the causes of sex-differentiation.

In Metazoa, fertilization is always selective, *i. e.*, between unlike gametes. Ovum does not fertilize ovum, nor do spermatozoa conjugate. It is true that a phenomenon known as fertilization by the second polar globule has been described in a parthenogenetic egg, but it is altogether improbable that it has the physiological value of fertilization. In Protozoa, also, fertilization is often selective, *i. e.*, between differentiated

gametes, and there are various degrees of differentiation from conditions essentially similar to the reproductive cells of Metazoa, to relatively slight unlikeness of gametes; and the latter grades into the conjugation of like gametes, which seems to be the primitive condition. It is almost universally believed that selective fertilization does not exist when the gametes are alike; any two gametes may unite. It, therefore, follows that the fertilization-need is the same in both gametes (even when they are differentiated). And from this idea arises the inconsistency between theories of the significance and the causes of sex differentiation; for if the gametes are in the same physiological condition, their differentiation, and sex differentiation itself, can only be devices to secure gametic union.

But there is an alternative point of view, viz., that fertilization may be always selective, even when there is no morphological gametic differentiation. I am convinced that only on such an assumption can a consistent theory of sex differentiation be constructed. If gametes be physiologically different, even when they are morphologically alike, then morphological differentiation of gametes follows naturally as an expression of these physiological differences, and sex-differentiation as a further stage in the same process of evolution.

Now Calkins has clearly demonstrated the probability that fertilization is selective even when the gametes are morphologically alike. He showed that, in *Paramœcium*, one of the ex-conjugants in each case has the greater vitality. He concludes: "This indicates that there is a physiological difference between *Paramœcium* gam-

etes analogous to that existing between egg and spermatozoon."

It would seem that the view that species have periodic phases of inefficiency, corrected by fertilization, is well founded. But it is difficult to see how the union of two like inefficiencies may restore efficient functioning. I have much sympathy with Weismann's strictures on the hypothesis that the union of two senescent beings may produce one rejuvenated being. But if we conceive fertilization as always selective, *i. e.*, between physiologically differentiated gametes, then the fertilization-need must be different on the two sides; and this may be conceived in one of two ways: either the gametes represent plus and minus deviations, respectively, from the physiological mean, in which event fertilization might be supposed to be a reciprocal process; or one gamete may be supposed to act as stimulus and the other as the element stimulated, in which event fertilization would not be reciprocal, but one-sided. Now, fertilization in ciliate infusoria has always been supposed to be a reciprocal process, and the morphological phenomena are all in favor of this point of view; but Calkins's results indicate that only one of the ex-conjugants is benefited; the fertilization is one-sided physiologically.

In either event, union in the zygote would restore the physiological mean or condition of equilibrium, and the question would arise, how the differentiated conditions are subsequently produced. Nothing definite can be said about this at present; but it is obvious that the protoplasm does tend inevitably away from the condition of equilibrium towards one or the other dif-

ferentiated condition; the direction of the tendency appears to be dependent on stimuli.

The objection may be raised that in some animals sex is certainly determined in the ovum at the time of fertilization, whereas, according to the point of view presented, fertilization is supposed to balance the physiologically differentiated conditions on which sex depends. Sexual determinateness of the fertilized ovum may, however, be interpreted to mean only that the sex-determining factors, primitively external, have been replaced by internal conditions in these cases. It is certainly not an illogical position that physiological neutrality in regard to sex may coexist along with internal conditions that absolutely restrict sexual differentiation to one direction.

In his thoughtful and suggestive paper on 'The Phenomena of Sex Differentiation,' Watasè comes to the conclusion that

> The organism is either a male or a female, not by the difference of primary sexual characters alone, but by the difference which saturates the whole of its entire structure. Such a difference is, however, neither absolute nor permanent. It is a temporary differentiation of protoplasm into one of two different directions, and sooner or later comes back to the original neutral or non-sexual state from which it started, thus manifesting the phenomenon characteristic of all protoplasmic irritability.

His point of view is instructive; there is a sexually indifferent stage of the organism corresponding to the period of union of the germ-nuclei; sexual differentiation is a phenomenon of irritability or response to stimulus, which lasts throughout the life history of the growing organism; 'and the recurrence of the irritable condition cor-

responds to the production of the unicellular embryo.' Sex differentiation is thus one of the phenomena of irritability, and it differs from other phenomena of this class only in the slowness of its rhythm.

Watasè's conclusions were based on the observations of Auerbach, himself and others, that the staining reactions of the egg and sperm nucleus are entirely different during the earlier stages of their existence, but that these differences disappear at the moment of fecundation. It has since been shown that the difference in staining reaction of the germ nuclei is probably of secondary significance only, but the view that a primary physiological difference between the germ-nuclei exists, is not necessarily excluded.

The question has arisen whether we are to deny the old biological conception of a sexually indifferent stage in the life history? It seems to me that this conception is as necessary and fundamental today as it ever appeared to be, and that we can not depart from it without involving ourselves in absolutely hopeless theoretical difficulties.

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ernment, the Carnegie Institution of Washington, the Wistar Institute of Anatomy and Biology, the Rockefeller Institute for Medical Research and the McCormick Institute for the Study of Infectious Diseases. These institutions recognize the fundamental importance of research for the well-being, nay, for the very life, of the commonwealth, and they also recognize cooperation as the vital principle in the conduct of research. The institution that inbreeds, that does not seek for the original and productive investigator, and that does not lend its own cooperation and secure his is on the high road to ineffectiveness.

I believe, however, that the full conception of cooperation in scientific research is not usually grasped and that the logical outcome of the principle is, therefore, not really understood: An organization may be formed that proposes to make cooperation with scientific men and institutions its main business; it may propose to seek out the original investigator wherever he may be found and to support his work in every possible way; it may welcome every new branch of scientific investigation and propose to favor it according to its importance and its needs; and yet such an institution may not be fully cooperative. It may be privately controlled; if so, its impulses are primarily benevolent and not free, guided by tradition and charter and not by the native interests of the governing body, and for these reasons apt to fail to profit to the fullest extent by the fertilizing influences of new conceptions.

The fundamental idea of cooperative organization is a free association of individ-

uals that proposes definite ends and effects an organization to attain them. The members of the organization are at the same time the court of last resort; they may elect representatives as a board of management, or as officers of the organization; but the representatives are responsible to the organization for the conduct of affairs. The functions of such an organization are not benevolent, but free, for the members are vitally interested in the conduct of its affairs and they are themselves the governing body. The organization is plastic, responding to new ideas, so long as membership in it is determined by broad principles and not merely by personal considerations.

Our scientific societies are the type of such organizations in science. The members make their own laws, elect new members, appoint officers in rotation from the membership, read their own papers and pay their own expenses. In turn, groups of members take on the duties of hosts to the others, and it is a genuine and deep pleasure, for are they not returning the numerous hospitalities that they have received? Such meetings are among the best events of the year in science, the most stimulating, the most fraternal; and the impetus to the progress of science is incalculable.

The Marine Biological Laboratory is another example of a free cooperative organization in biology. I believe I need not apologize for using this organization to illustrate my remaining remarks, because it belongs to the Naturalists of America and it illustrates better than any other in-

stitution with which I am thoroughly familiar certain working principles of co-operation in biology.

The government of the laboratory is vested in a corporation consisting of 64 life members and about 125 annual members, and a board of trustees of 31 members (at present, full number 35), of whom three are *ex officio*. The corporation meets annually to elect a clerk, treasurer and eight trustees to serve four years. Membership in the corporation is by election by the board of trustees. To the latter body is delegated the administration of laboratory affairs, and they appoint all officers and agents. Of the 189 members of the corporation, 129 are professional biologists; of the 31 members of the board of trustees, 25 are of the same class. The corporation has the power to modify the policy of the laboratory in any respect by virtue of its control of the membership of the board of trustees.

The government and ownership of the laboratory thus rest absolutely in the hands of American biologists, and this is the first and fundamental cooperative feature of the organization, viz., cooperation of those interested in affecting an organization for research. I believe that much of the usefulness and effectiveness of the institution rests on this basis. It establishes a thorough democracy of sentiment, which is more nearly like the sentiment of our scientific societies than that of any other institution that I know. Out of this grows a feeling of loyalty that is shared in greater or less degree by nearly all who have worked at the institution. These now

represent practically every university and college of the country, most of which are represented either in the board of trustees or in the corporation.

This fundamental cooperative principle was established at the foundation of the laboratory, though the proportion of professional biologists in the membership, both of the corporation and of the board of trustees, was less then than now.

A second important form of cooperation exists between the laboratory and other institutions. Many of our institutions require the facilities of a marine station for the research work of their biological departments. Some have sought to supply these facilities independently, but I believe I am correct in saying that this method has not usually proved permanently successful, partly because of the expense of the undertaking, and partly because such an individual undertaking lacks the inspiration that comes from the presence of representatives of other institutions, who bring in new ideas and establish a congenial community.

In the year 1891, after four years of experience in conducting the affairs of the laboratory, a committee of the board of trustees prepared and sent out a circular letter requesting the cooperation of the biological departments of our colleges and universities in the maintenance of the Marine Biological Laboratory. The form of cooperation suggested was that of subscription for students' tables or research rooms, the former at $50 each and the latter at $100 each for the season. In this way the laboratory anticipated the need of

biological departments for marine facilities both in instruction and in research.

The response was unexpectedly general and prompt; the following institutions subscribed at once: Columbia, Brown, The Missouri Botanical Garden, Williams, Chicago, Massachusetts Institute of Technology, Rochester, Bryn Mawr, Mount Holyoke, Vassar, Wellesley, Cincinnati, Miami and Northwestern. The majority of these institutions have continued their subscriptions up to the present time. Others have since come in, some sporadically, others continuously. Last year the number of subscribing institutions was 17. There has not, however, been considerable growth in this respect; and this is perhaps partly due to the fact that the laboratory has rarely refused a free working place to competent applicants, unless space was lacking.

It is clear that an institution may secure for its investigators by this form of cooperation the best of facilities for marine work at a cost many times less than would be required on an independent basis, with the added advantage of association with representative investigators from other laboratories. At the same time such an institution is aiding to support an organization that supplies one of the most general needs of American biology.

Special forms of cooperation with institutions are entered into from time to time. Relations with the Woods Holl Station of the U. S. Bureau of Fisheries have always been mutually helpful. The laboratory also exchanges investigators' tables with the biological stations of Canada and de-

sires to enter into similar relations with other marine laboratories. For three years the laboratory furnished working places for twenty appointees of the Carnegie Institution and this relation was a great aid to the Marine Biological Laboratory at a critical juncture of affairs, and furnished a permanent stimulus and incentive to the prosecution of its work.

It is very desirable that such cooperative relations with other institutions should be extended and strengthened; and it is probable that this will take place in the near future. The maintenance of such relations implies that they shall be mutually advantageous. I believe that this has been demonstrated, and that, in proportion as this is realized and the spirit of research increases in our institutions, such cooperative relations are bound to grow. The principle of cooperation does not mean that all shall do the same amount, but that all shall enter into it in the same spirit and do according to their means and opportunities.

A third fundamental form of cooperation is that of and with the various subdivisions of biological inquiry. Four departments are formally organized in the Marine Biological Laboratory; in the order of their establishment they are: zoology, botany, embryology and physiology. These are, of course, broad divisions and their organization as departments does not mean that other subjects are excluded; indeed, the laboratory welcomes any biologist with a problem in the solution of which the facilities or fellowship of the organization may aid. There has been a good deal of

research done both in pathology and in psychology at the laboratory, and it is hoped that it will grow; the laboratory is ready when the time is ripe to organize departments in these subjects.

The problems in the various departments of biological research are so intricately interrelated that each department is sure to be of aid to others in many ways often curious and unexpected. This is not a matter for surprise. It only emphasizes the necessity of the broadest organization of our work if any subject is to march forward with the least degree of impediment.

Indeed, I question very much if the work can logically be confined to the subject-matter of biology. Our physiological chemists are already urging a chemical laboratory. This seems necessary for the physiologist who has chemical problems, and it may prove attractive to the chemist who has biological problems. A department of chemistry in connection with the Marine Biological Laboratory would be of distinct advantage to every other department; even the morphologist has his chemical problems, particularly in connection with the complex matter of stains.

An institution organized in the manner outlined is in a position to develop in harmony with its environment, and should be in little danger of outliving its usefulness, because it is national, representative and plastic. It should thus represent at any time the best traditions and methods of research so long as it maintains the fundamental forms of cooperation: its free organization, its free cooperation with sci-

entific societies and men, and the free cooperation of the biological sciences within the common organization. If these are maintained, it must inevitably keep pace with the increasing subdivision of biological specialties and be guided by the community of ideas and problems in different fields.

An important result of the cooperation of biologists in the maintenance of the Marine Biological Laboratory is the direction given to research. It is true, of course, that every productive investigator aids in giving direction to research; he has students and he has imitators. Where a number of such investigators come together for considerable periods of time and problems are discussed, there tend to be a clearing of ideas and sharper definition of problems. This effects a larger circle, and the influence spreads. The best results of this sort can be attained only if people discuss their work freely, and do not keep it locked up until after publication. I believe that a considerable effect has been thus produced in direction of research, particularly in cytology, experimental embryology and physiology at the Marine Biological Laboratory. Organized efforts to direct the research of others, excepting one's own students, are apt to be futile, especially on a large scale, and in a large subject such as biology, where fundamental problems are dimly perceived or perhaps yet unsuspected. For this reason and others even more important, the institution has never prescribed or limited subjects of research.

I have spoken thus for the most part

about the work of the Marine Biological Laboratory, because it illustrates in a practical working model various possible forms of cooperation; and because such a demonstration is worth much more than a merely academic exposition of the nature and advantages of cooperation.

FRANK R. LILLIE

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On the Specific Gravity of the Constituent Parts of the Egg of Chætopterus and the Effect of Centrifuging on the Polarity of the Egg (Abstract): FRANK R. LILLIE, University of Chicago.[1]

As the result of experiments with centrifugal forces of graded powers, the author concluded that the protoplasm of the polarized ovocyte of *Chætopterus* possesses both a polar and a concentric organization of its *ground substance*, which is the basis of the so-called "segregation pattern," or arrangement of granules ("substances") within the egg, and which determines the axis of embryonic development. This organization of the ground substance is not substantially altered by centrifuging, though it may be temporarily distorted thereby.

These conclusions depend upon the following considerations and observations:

I. *Concentric Organization.*—If the ground substance be perfectly homogeneous, granules moving under the influence of centrifugal force will pass in straight lines centrally and distally, according to their respective specific gravities, and will not be held in any intermediate position. Aggregation of granules in definite configurations or inter-

[1] Read before the joint session of the American Society of Zoologists and Section F—Zoology—of the American Association for the Advancement of Science, Chicago, December, 1907.

mediate positions after centrifuging are *prima facie* evidence of differences of resistance of the ground substance.

Such definite configurations are found in the egg of *Chætopterus* after the action of low centrifugal powers with perfect constancy; and these together with the evidence derived from study of the normal structure enable us to infer the following concentric organization of the ground substance: (1) An ectoplasmic layer of considerable density, open at the animal pole; (2) an intermediate more fluid layer which is especially developed at the animal pole, where it comes to the surface; (3) a ring of greater density, enclosing (4) a large subcentral, more fluid mass. The last corresponds in position and approximately in size with the original location of the large germinal vesicle.

As a consequence of this organization of the ground substance, the small basophile granules within the central, more fluid area aggregate in the dense ring bounding it, when low centrifugal powers are used. This aggregation is situated towards the axis of the centrifuge, as the basophile granules are of low specific gravity, and is lunate in cross-section; it contrasts vividly with the surrounding parts of the egg after staining in strong basic dyes, such as thionin. Higher powers of the centrifuge drive such granules through the dense ring into the "clear zone" which contains the basophile granules from other parts of the egg. The acidophile granules also show typical configurations. The central, more fluid mass is practically emptied of granules when low centrifugal powers are used, because those within it are driven into the

dense ring bounding it and those without do not penetrate the dense ring to enter it.

II. *Polar Organization.*—The polarity of the ovum before and after centrifuging appears to be the same whether the karyokinetic spindle retains its original position or not. This is inferred from the fact that the polar axis of centrifuged eggs (as determined by the position of the polar bodies appearing subsequent to centrifuging, and the later development) bears no constant relation to the axis of stratification of granules produced by centrifuging. Therefore the position of the granules of the egg does not determine its polarity. The latter may, therefore, be either a persistent feature of the ground substance, or may be determined by whatever point on the surface the maturation spindle happens to reach if displaced by centrifuging. The latter proposition appears highly improbable because (1) the polarity of ovocytes with intact germinal vesicle does not appear to be determined by the point on the surface to which the germinal vesicle may be thrown by centrifuging (limited number of observations of author); (2) the position of the maturation spindle displaced by centrifuging is constant, viz.: in the clear band, but the polar bodies are frequently formed outside this band; (3) in other animals no cases are known where the position of the nucleus has been shown to determine polarity, but, on the contrary, the polarity of the ovum is known to determine the position assumed by the cleavage nuclei. Therefore, it is believed that in *Chætopterus* the ground substance retains its polarity after centrif-

uging and that the place of formation of the polar bodies is thereby determined.

III. As to specific gravity of the cell contents, the so-called gray cap, which is specifically lightest, is made up of residual substance of the germinal vesicle and numerous small granules that are blackened by osmic acid and are soluble in xylol, hence presumably fat granules. The intermediate or clear band contains the majority of basophile granules; the distal hemisphere contains the acidophile granules, and of these, those originally contained in the ectoplasm appear to be heaviest.

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A Contribution towards an Experimental Analysis of the Karyokinetic Figure (Abstract): FRANK R. LILLIE, University of Chicago.[1]

The alternative hypotheses (1) that the poles of the karyokinetic figure (centrosomes) are centers of force which acts outwards and exerts its effect at a distance (centrosome theory) and (2) that the centrosomes are merely centers of insertion of the organic radii (mitome theory), may be tested experimentally. On the first hypothesis, spindles that move through the protoplasm would produce new asters in the form of radiating "chains of force" (Hartog); on the second hypothesis the moving spindle would drag the organic radii with it and produce considerable distortion of the karyokinetic figure. On the first hypothesis, not only the rays of the asters, but also the fibers of the spindle, must be interpreted as chains of force, and the chromosomes as well as centrosomes may be centers of force. Therefore, in any movement of the spindle through the cytoplasm, it is conceivable that its material also is constantly changing. If, therefore, the karyokinetic figure were suddenly moved from protoplasm of one morphological character into protoplasm of another, comparison of the normal and

[1] Read before the joint session of the American Society of Zoologists and Section F—Zoology—of the American Association for the Advancement of Science, Chicago, December, 1907.

experimental conditions of the karyokinetic figure would furnish evidence for deciding between the two hypotheses.

The unfertilized eggs of *Chætopterus* enable us to make such a test. After they have remained in sea water fifteen to twenty minutes, each possesses a large karyokinetic figure fixed at the animal pole, and this figure remains at the mesophase for hours. If such eggs be centrifuged with relatively low powers (1,150 revolutions in 30 seconds to 1,750 revolutions in 45 seconds) not only may the karyokinetic figure be moved through the cytoplasm, or the latter caused to flow past the spindle, but new configurations arise in the cytoplasm, owing to central or peripheral displacement (with reference to the direction of the centrifugal force) of the granules suspended in the ground substance of the cytoplasm. The most important of these configurations for our purpose is a dense accumulation of the basophile granules a little central to the middle point of the egg. This is a new formation called suddenly into existence, within 30 to 45 seconds, and contrasting strongly with the remainder of either normal or centrifuged eggs.

If a pole of the spindle enter this basophile mass, the granules of the latter appear to be rearranged in radiating lines that can be interpreted only as chains of force. Apparently the original radiations of the pole in question are lost, or are re-enforced. The rays of the aster in the basophile mass are actually more numerous than in the karyokinetic figures of control eggs, as determined by counting.

Any part of the basophile mass that enters the spindle appears also to be rearranged in chains of force: one finds that the part of the spindle within the basophile mass usually stains correspondingly, and thus contrasts strongly with the part without, and this is probably due in the first place to infiltration of basophile granules; but the modified part of the spindle is also fibrous in its structure and the interfilar spaces are empty of granules, so that it would appear that the infiltrated granules are arranged in chains.

Such distortions as are found in either the rays of the aster or the fibers of the spindle are explicable on the chain of force hypothesis; because it has been shown by Hartog that chains of force consisting of iron dust suspended in glycerine or balsam will bear great distortion before rupturing; and this is due in the first place to the fact that the longer path of a distorted chain is more permeable than the relatively resistant medium in which the permeable chains of particles are embedded, and in the second place (to a certain extent) to the viscosity of the medium.

The mitome hypothesis appears inadequate to explain the results of the experiments, and may certainly be regarded as disproved if actual counts show a definite increase in the number of astral rays proceeding from the pole embedded in the basophile mass.

If the center of force hypothesis be accepted, the results show that the basophile granules are extremely permeable to the "mitokinetic force" (Hartog) and that the ground substance is highly imperme-

able. Acidophile granules are also impermeable; or at least they are not arranged in chains of force.

POLARITY AND BILATERALITY OF THE ANNELID EGG. EXPERIMENTS WITH CENTRIFUGAL FORCE.[1]

FRANK R. LILLIE.

In a previous paper (Lillie, '06) I attempted to show that the direction of polarity of the egg of *Chætopterus* is not modified by any experimental redistribution of the visible elements, nucleus and granules, and I therefore concluded that polarity is a property of the ground substance of the protoplasm. In the present paper I propose to examine the grounds for this statement, to attempt to show that bilaterality comes in the same category, and to examine the conceptions of polarity and organization that naturally result.

I. Polarity.

Polarity manifests itself first in the egg of *Chætopterus* when the ovogonium becomes an ovocyte and takes its place in the ovarian epithelium. It has then a free pole and an attached pole, which have different physiological relations and exhibit different morphogenic activities. When the egg becomes free from the epithelium it is found that the original free pole becomes the animal pole in development, and the attached pole becomes the vegetative pole. The developmental processes take place with reference to these poles; thus the various granules of the egg rearrange themselves in a definite polarized fashion, the polar bodies have an absolutely determined relation to polarity, cleavage takes place and the various organs arise in definite topographical relations to the polarity.

In the paper already referred to I showed that if the egg is centrifuged with sufficient force the result is to separate the protoplasm into three strata, viz.: a central grayish cap, an intermediate hyaline or clear band which contains the nucleus

[1] The substance of a part of this paper was presented before the joint session of the American Society of Zoölogists, and Section F— Zoölogy — of the American Association for the Advancement of Science, held in Chicago, December, 1907. (An abstract was printed in *Science*, Vol. XXVIII., No. 702, pp. 905–907, June 12, 1908.)

or spindle, and a distal yellow hemisphere which contains the larger granules known as yolk (Fig. 1). The results prove that the eggs are not oriented in the centrifuge with reference to the original polarity, and therefore the axis of stratification bears all

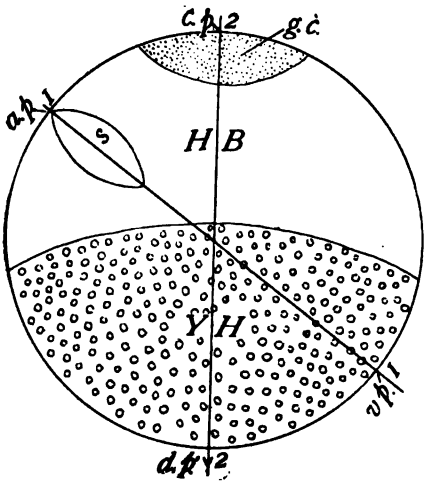

FIG. 1. Diagram of a centrifuged egg of *Chætopterus*. *a.p.*, animal pole; *c.p.*, central pole, or pole turned towards the axis of the centrifuge; *d.p.*, distal pole, or pole turned away from the axis of the centrifuge; *g.c.*, gray cap; *H.B.*, hyaline or clear band; *s.*, maturation spindle; *v.p.*, vegetative pole; *Y.H.*, yellow, yolk-bearing, or distal hemisphere; *1–1*, primary axis; *2–2*, secondary axis.

possible relations to the polar axis of the egg. We may call the original polar axis the primary axis, and the line through the center of the egg in the direction of the centrifugal force the secondary axis (Fig. 1).

If such eggs are fertilized the polar globules of different eggs form at any point of the surface with reference to the secondary axis. Cleavage takes place with reference to the axis defined by the polar globules, as in the normal egg, and is approximately normal in form. It therefore follows that the distribution of yolk and other substances induced by the centrifuge is substantially immaterial so far as the form of cleavage is concerned. The effect of abnormal distribution of yolk and other substances on the differentiation of organs is not considered here.

On the basis of these results I concluded that the polarity of the ovum had not been affected by centrifuging, and that the

polar bodies appear in the same position that they would have normally, that is, in the primary axis; in other words, that no redistribution of the granules or nucleus affects the direction of polarity of the egg. If this be true, then the final conclusion stated in that paper, that polarity is a function or property of the ground substance of the protoplasm, certainly follows.

This conclusion is of fundamental importance for the entire theory of development, and its basis therefore requires the most careful examination. One thing is clear at the start, viz.: that this point of view affords a complete explanation of the phenomena, because the egg is polarized prior to centrifuging, and the centrifugal force acts in all possible directions with reference to the polar axis;[1] and this agrees with the fact that the polarity that appears after centrifuging also lies in all possible directions with reference to the axis of stratification. The question is simply this: Is the polarity as marked by the polar bodies and cleavage planes the same polarity in the normal egg and after centrifuging? Does any other hypothesis agree so well with the observed facts, and if so, is it for any sufficient reason to be preferred to this hypothesis?

An alternative hypothesis can lie only in one direction, viz.: that polarity is determined by any point on the surface of the egg to which the maturation spindle happens to go after cen-

[1] The proof of this statement is as follows: In the stage used for the experiments (except where otherwise stated) the first maturation spindle is fixed at the periphery at the stage of the mesophase. Its outer pole marks the center of the animal pole; this point is also characterized by an entire absence of the ectoplasm, which covers the remainder of the surface of the egg as described in a previous paper ('06). If sections of centrifuged eggs are examined, the maturation spindle is found in about half of the eggs still attached to the surface, at any point in the hemisphere containing the hyaline band. At first sight this would indicate that the eggs oriented themselves in the centrifuge to a slight extent, though it is clear that the animal pole is in some cases 90° removed from the ends of the secondary axis. But the fact that fixed spindles are not found in the distal (yolk-laden) hemisphere after centrifuging has another explanation: about half of the eggs after centrifuging have free spindles (*i. e.*, not attached to the surface), which always lie in the hyaline band, and these represent those eggs whose original animal pole lay in the distal hemisphere in the centrifuge. (Cf. Fig. 8, *C* and *D*.) The inrush of yolk into the distal hemisphere has torn the spindles loose from the periphery, and their specific gravity has carried them into the hyaline band; this interpretation of the free spindles is proved by the fact that many of them migrate into the distal hemisphere later in the process of forming the polar bodies (Fig. 2).

trifuging. Now, assuming that there is no polarity of the ground substance, as this hypothesis requires, the spindle would naturally follow the shortest path to the surface, for this would usually be the path of least resistance. In other words, the position given the nucleus or the spindle by the centrifugal force would determine the polarity in all cases, because its position is always excentric. However, this is not the case, as the following facts demonstrate :

1. Most of the experiments on the egg of *Chætopterus* were made before fertilization at the stage of the mesophase of the first maturation spindle. All the ripe eggs of this animal attain this stage without fertilization, and the spindle comes to rest and remains at the mesophase unless the egg be fertilized or otherwise effectively stimulated (Fig. 4). The spindle is attached to the surface and its axis is in line with the axis of the egg. If the direction of the centrifugal force is such that the spindle lies in the central hemisphere, *i. e.*, in the half of the egg towards the post, it may remain attached, but is often detached from the surface, and in such cases it always lies in the clear band. If the spindle pole of the egg lies in the distal hemisphere during centrifuging, it is always detached from the surface and comes to rest in the clear band. The result therefore is always the same so far as the position of the spindle is concerned : the spindle lies invariably in the clear band after centrifuging, either attached to the surface, or free.

Nevertheless, the polar bodies may form at any point in the yolk hemisphere, as is demonstrated by study of the living eggs, and also by sections of series of eggs preserved during the maturation period after centrifuging. Figures of such living eggs are given in the paper already referred to, and a section of such an egg is reproduced here (Fig. 2). When one considers the resistance that has to be encountered by the spindle in making its way through the densely packed yolk-granules of the distal hemisphere, and remembers that a shorter path to the surface through hyaline protoplasm was open, it is obvious not only that the spindle does not follow the shortest path to the surface, but also that a factor of great intensity has been in operation.

I stated in my previous paper ('06) that the formation of polar

globules in the yolk hemisphere is rarer than their formation in the hyaline hemisphere, and was inclined to regard this as evidence that there was a tendency for the eggs to orient themselves in the centrifuge with the vegetative pole away from the center. But I no longer believe that this is the case. The relative rarity of maturation at the distal pole of centrifuged eggs must be due to a different cause, and this is the great resistance of the yolk

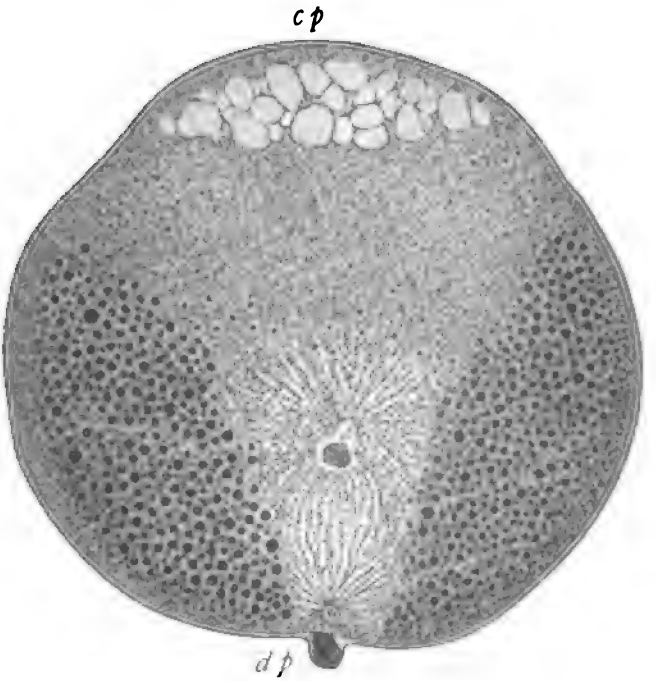

FIG. 2. Egg of *Chætopterus* centrifuged 2,300 revolutions in one minute at a radius of 13 cm., 3 : 57 P. M., June 27, 1906; fertilized 3 : 58 P. M., killed 4 : 27 P. M. The formation of the polar globules has taken place at the distal pole. The maturation spindle migrated through the entire yolk-mass in the distal hemisphere to reach this position. The sperm-nucleus surrounded by radiations is seen a short distance above the egg-nucleus. The vacuolated area above is the gray cap. *c.p.*, central pole (secondary axis); *d.p.*, distal pole (secondary axis).

mass to penetration by the spindle. This conclusion is clearly demonstrated by study of series of sections of eggs preserved at regular intervals after centrifuging and fertilizing, which show that many such eggs fail to form the polar bodies at all, or are much delayed, or undergo their maturation divisions within the egg.

2. An even more conclusive argument is afforded by centrifuging ovocytes with intact germinal vesicle. As shown in the paper already referred to, the large germinal vesicle passes to the central pole; it is the lightest constituent of the egg. If, therefore, the position of the nucleus determines polarity, the central pole should become the animal pole in such cases; the polar bodies should form here. I made a number of experiments on this subject in 1906 and 1908. The difficulties found in interpreting these experiments are: (*a*) that the stratification induced by low centrifugal powers is relatively slight in the stage with intact germinal vesicle, apparently owing to greater vicosity of the ground substance at this stage, and (*b*) that it does not persist to the time of formation of the polar bodies, owing to the process of polarization of granules that follows the rupture of the germinal vesicle, which I described in a previous paper. However, by following individual eggs in which the position of the centrifuged germinal vesicle is known, the relation of the polar bodies to the position of the germinal vesicle can be readily ascertained. It is then found that there is no fixity of relation whatever; in other words, the maturation spindle, which forms at the site of the germinal vesicle, may migrate through the entire diameter of the egg to become fixed at the opposite pole, or perform any lesser migration. The same thing is demonstrated if the eggs are submitted to very high centrifugal powers; under these circumstances the stratification persists up to maturation in some eggs to a sufficient degree to demonstrate positively that the polar bodies form in all positions with reference to the centrifuged position of the germinal vesicle.

Thus one cannot determine the place of formation of the polar bodies by simply throwing the nucleus to the surface of the egg.

A third argument may be based on comparative grounds. Very many cases are known in which polarity determines the position of the nucleus; the literature on fertilization is full of such cases. I know of no observations that tend to show determination of polarity by position of the nucleus.

The results, then, certainly demonstrate that polarity is not a result of the position of the nucleus or of any configuration of granules. It follows that it is a property of the ground sub-

stance; and when one considers carefully the data presented, one cannot avoid the conclusion that the direction of polarity is unmodified by the centrifugal powers employed. It must therefore depend on some configuration or heterogeneous physical or chemical properties of the ground substance established early in the history of the egg, and which is not essentially disturbed by centrifuging. This subject will be examined in the third part of the present paper.

The conclusion stated above was clearly presented in my paper of 1906. Since then Morgan (1908) has come to the same conclusion for the egg of *Arbacia*. In his last paper Morgan furnishes a satisfactory demonstration that polarity is unmodified in the egg of *Arbacia* by centrifuging, by showing that the micropyle which bears a constant relation to polarity in the normal egg has the same constant relations to the polarity of centrifuged eggs, as proved by formation of the micromeres and archenteron at the pole opposite the micropyle whatever the direction of the centrifugal force, and therefore whatever the position of the centrifugal zones and the form of the cleavage modified (in *Arbacia*) by these zones. I therefore take Morgan's result to be a complete confirmation of my original and present position.

The demonstration that the direction of polarity is unaltered by centrifuging involves the assumption that the configuration of the ground substance remains the same. In other words, that there is a definite architecture in the ground substanee, which is the basis of the localization pattern in normal development (see *Science*, N. S., XXVII., June 12, 1908). This also involves a new conception of protoplasmic streaming as seen in ova, for this must be interpreted as granule movement exclusively, and not actual flowing. Similarly, the movements produced by the centrifuge cannot be mass movements of entire protoplasmic areas, but only granule movements through the ground substance. In the third part of the present paper, I shall consider these conclusions with some care.

Observations on Nereis.

In the summer of 1908, I had the opportunity of comparing the eggs of *Nereis limbata* with reference to these points, and I

found that, just as in *Chætopterus*, the place of formation of the polar globules is independent of centrifugal stratification, and that the cleavage follows the polarity marked by the polar globules and is not modified in its direction by the position of the yolk arbitrarily imposed by the centrifuge. Morgan has shown the same thing for the lamellibranch, *Cumingia* (*Science*, Vol. XXVII., p. 496, 1908), so that now we have at least four forms in which this principle is firmly established.

II. Bilaterality.

The second main question that I propose is whether or not bilaterality comes under the same category as polarity. And this resolves itself into two questions: (1) Is bilaterality, like polarity, a function of the ground substance, and (2) When does it arise?

1. The first morphological evidence of bilaterality in the eggs of *Chætopterus* and *Nereis* is found in the first cleavage. The spindle takes an excentric position, and the cleavage is therefore unequal; the smaller cell is anterior and the larger cell posterior in position. The course of the entire development is thus determined not only with reference to the bilateral axis, but also with reference to the proportions of embryonic organs.

If the bilaterality were a result of a kind of equilibrium of the protoplasmic inclusions (nucleus and granules), it is clear that the variations in position of the nucleus and granules produced by centrifugal force should involve change of direction of bilaterality. If, on the other hand, bilaterality is a function of the ground substance, changes in the position of the nucleus and granules should not materially affect the direction of bilaterality, though it might conceivably modify the inequality of the cleavage which is our index of bilateral organization. However, if the normal proportions of the first two cells and the meridional direction of the cleavage plane are maintained in centrifuged eggs in spite of the abnormal distribution of granules between cells that would be thus induced, no other evidence would be necessary to show that the seat of bilateral organization is in the ground substance.

This is in fact the case, both in *Chætopterus* and *Nereis*: Whatever the distribution of yolk and granules induced by centrifuging,

with reference to the primary axis, the first cleavage is invariably truly meridional, and the proportions of the cells are usually normal or approximately so. In some cases the small cell is packed full of yolk granules, in other cases practically devoid of them; nevertheless, the first cleavage is substantially unaffected as to direction or proportions by the direction of stratification (see Lillie, 1906, p. 199).

2. Is the bilateral organization of the egg a property of the primary ovocyte like polarity, or does it arise in the course of development after rupture of the germinal vesicle? The evidence seems to me in favor of the latter proposition for the following reasons:

(*a*) If the ground substance is bilaterally organized before maturation there should be morphological evidence of it, as there is of the existing polarity. But there is none whatever; the structure of the egg is radially symmetrical in the plane at right angles to the polar axis until the close of maturation. After the union of the germ nuclei, the bilateral symmetry comes to expression slowly by granule movements that cause a preponderant distribution of the yolk granules on one side of the egg, which becomes the posterior side, and by corresponding displacement of the yolk-free protoplasm and spindle towards the opposite side of the egg, which becomes the anterior side. This is the case in both *Chætopterus* and *Nereis*, as well as in *Unio*. If the conditions causing such displacement existed prior to maturation, one would expect that they would become effective at an earlier period.

(*b*) In the summer of 1908 I found that it was possible to break up the eggs of *Chætopterus* into fragments of varying sizes by high centrifugal force. The protoplasm of this egg is much more fluid than that of any other egg I have tested; in consequence, the egg elongates even with relatively low centrifugal speed; and with very high speed the eggs break apart along the lines of stratification. Thus one obtains numerous perfectly hyaline pieces composed of the substance of the clear band, and others densely packed with yolk granules. The nucleus is almost invariably contained in the former kind, but it may occur in the latter, owing to the fact that at the time of centrifuging the matu-

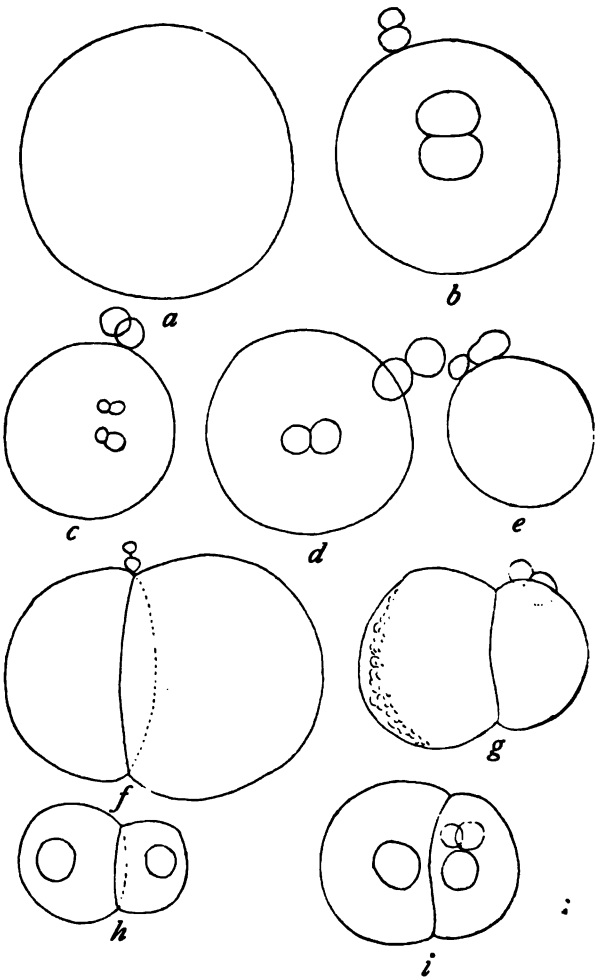

FIG. 3. Maturation and first division of hyaline fragments of the eggs of *Chætopterus*, obtained by centrifuging before fertilization. *a*, outline of normal egg for size-control; *f*, outline of normal two-celled stage for size-control; *b*, *c*, *d*, *e*, *g*, and *h*, hyaline fragments of various sizes from a single experiment; *i*, a similar fragment from another experiment. Fragment *g* contains a small quantity of yolk.

The eggs break apart along the planes of stratification with a high centrifugal force, and further subdivision may occur so that the fragments are variable in size.

It will be noted that the polar globules are abnormally large in the fragments (cf. Fig. *f*, control). The proportions of the first two cells are, however, approximately normal, Figs. *g*, *h*, *i* (cf. *f*, control).

All figures drawn with the camera at a magnification of 780 diameters, and reduced one half.

ration spindle is attached to the surface, and may thus be separated off in the yolk-bearing end of the centrifuged egg, if it lies in a distal direction in the centrifuge and is not torn loose from its attachment before rupture of the egg takes place. But while the majority of large hyaline pieces contain the maturation spindle and form polar bodies after fertilization, this happens very rarely indeed in yolk-bearing pieces.

I was interested to determine if the cleavage of the hyaline pieces was unequal, and, if so, whether or not the proportions of the cells approximated the normal. This is indeed the case; in most pieces taken before fertilization, that segment, the segmentation is unequal and in substantially the normal proportions. Seeing that the inequality of cleavage of the normal egg is an unfailing index of bilaterality, the same phenomenon in the parts must be interpreted in the same way.

The unequal cleavage of such hyaline parts absolutely devoid of yolk is a most striking phenomenon. There is great variation in the absolute size of such parts, but the cleavage remains proportional.

The parts vary not only in size, but also in the regions of the egg that they represent. The latter conclusion obviously follows from the fact that the direction of the centrifugal force is a matter of chance.

To show the strong tendency of pieces of the hyaline band to segment in normal proportions the following result may be cited. In experiment 10 *F*, August 18, 1908, a quantity of eggs taken from the female at 10 A. M. was allowed to stand in sea water without fertilization until 10:40 A. M. They were then centrifuged about 7,500 revolutions in one minute with a radius of 6 cm. and were thus broken up. The material was fertilized at 10:43 and allowed to develop to the two-celled stage, then fixed, stained and mounted entire in balsam. The impression of the preparation is that in practically every segmented hyaline piece the proportions of the cells are approximately normal. To remove the personal equation as far as possible, I marked a ring on the cover slip, and drew every segmented hyaline piece in it. Of 26 pieces so drawn, none had divided equally; in four pieces of the 26 the small cell was proportionally larger than normal,

and the remainder were substantially normal in the relative sizes of the two cells, although the smallest piece was about one eighth the volume of the entire egg, and the others ranged in size up to about one third the bulk of the normal egg. The pieces in which the small cell was too large relatively were the largest pieces and contained some yolk.

Now if bilateral symmetry existed prior to fertilization, one would expect parts of the bilateral egg to vary greatly in the proportions of the first two cells, because a fractional bilaterality and not a whole bilaterality would exist in each part. I therefore conclude that bilaterality has developed subsequent to fertilization. And this conclusion must be true *for the parts* even if it were only a question of regulation of a preëxisting bilaterality (of which there is no evidence). But if bilaterality can develop in the parts there is no reason for assuming its prior existence in the whole; and its origin must be regarded as a truly epigenetic process. Brachet makes a similar argument for the egg of the frog (*Roux's Archiv*, XXII., 3, 1906).

III. The Organization in the Ground Substance. Examination of the Conceptions of Polarity and Bilaterality.

The hypothesis that the nature and direction of polarity and bilaterality are unaltered by centrifuging involves the assumption that the arrangement of the ground substance is substantially unaltered. If it could be shown, for instance, that the segregation produced by centrifuging is a mass movement of the areas and zones of the protoplasm, and not simply a movement of granules through the ground substance, the entire organization of the egg would be altered, and there would be no reason why polarity should appear in the original direction rather than in any other direction.

I propose therefore to examine the thesis that the movements produced by centrifuging are purely granule movements, with the single exception of the nucleus or spindle. To do this it is necessary to compare minutely the structure of the egg both before and after centrifuging, using a fixed and determinate stage for such examination. Fortunately, the latter condition is a rela-

tively simple matter in the case of the egg of *Chætopterus*, because all ripe eggs reach such a stage in about twenty minutes after they are put in sea water, and undergo no further progressive development unless fertilized or otherwise suitably stimulated.

This stage may be described as follows (Fig. 4):

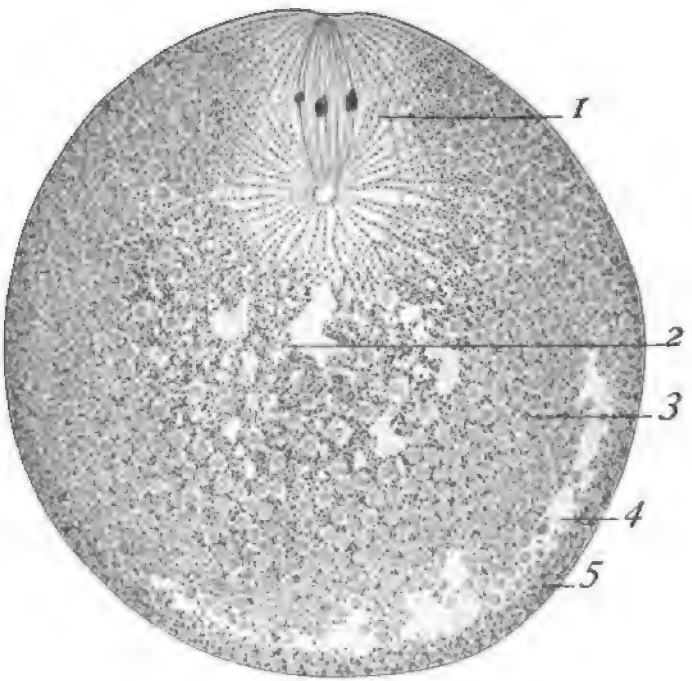

FIG. 4. Section of the egg of *Chætopterus* at the stage used for the experiments. See text for description.

The substance of the polarized egg shows the following areas and zones:

1. *The spindle area* lies at the animal pole and includes the spindle and the protoplasm in which the astral rays are developed. This area is predominantly basophile, but it may include a very few acidophile yolk granules. The astral rays may encroach slightly on (2) and (3).

2. *The central spongy area* lies near the center of the egg in the direct line of prolongation of the spindle. Its spongy appearance is due to paucity of granules and not to actual vacuoli-

zation of the ground substance. The basophile granules predominate and are arranged in a loose spongy network. This area is bounded by the spindle area on the animal pole side, and by the spherular zone (3) on the other sides. The boundary towards the spindle area is a little indefinite, fairly definite on the other sides. Its form is that of an indented sphere, the indentation being produced by the spindle area. Its position is about the same as that of the germinal vesicle before rupture.

Together, the spindle area and the central spongy area form an ovoidal mass, the small end of which reaches the surface at the animal pole, while the large end extends below the center of the egg.

The other zones of the egg are arranged concentrically to this mass, and are therefore open at the animal pole and thickest at the sides and vegetative end.

3. *The spherular zone* is so named because it contains the major part of the endoplasmic spherules or endoplasmic acidophile granules (yolk). In section, it appears as a very deep gourd-shaped crescent. The thin horns reach up above the level of the equatorial plate of the spindle.

4. *The intermediate spongy zone* lies between (4) and (5). In many eggs it is barely noticeable, in others it is very prominent. Like the central spongy zone, it is not a vacuolated portion of the ground substance, but it is characterized by paucity of granules, and so appears unstained in sections. It is a genuine structural feature as is proved by experiments, and also by the fact that it becomes more pronounced in eggs allowed to remain in the sea water (Fig. 5).

5. *The ectoplasmic zone* is the peripheral boundary of the egg, except that it is defective at the animal pole where the spindle area comes to the surface. It is characterized by the presence of spherules that differ in certain properties from those of the spherular zone (endoplasm), as described in detail in a previous paper.

In eggs allowed to stand in sea water without fertilization these areas become more strongly differentiated (Fig. 5), and the fact that the egg has a concentric as well as a polar organization stands out more strongly. The central spongy area now appears

to have a thick and dense wall in which the spherules of the spherular zone are especially aggregated. The intermediate spongy zone appears wider. The striking similarity of this con-

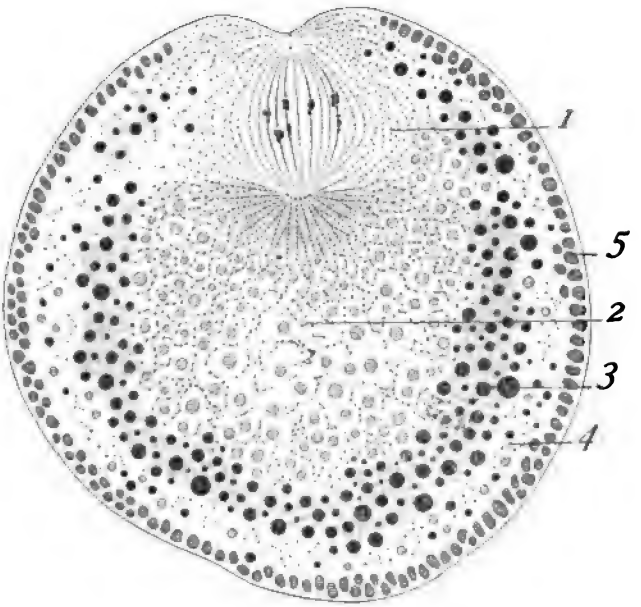

FIG. 5. Section of an unfertilized egg of *Chætopterus* that stood in sea water two hours and seven minutes before killing.

figuration to that produced by low centrifugal powers will be seen as the description proceeds.

We may now compare the pictures presented by eggs of this stage that have been centrifuged at different speeds: For this purpose we shall select first an egg that was centrifuged 1,150 revolutions in 31 seconds at a radius of 13 centimeters (Fig. 6). Comparison of a large number of eggs shows first that the position of the eggs in the centrifuge is a matter of chance and second that the segregation produced is essentially the same in whatever direction the centrifugal force may act with reference to the polar axis. We may therefore leave the question of direction of centrifugal force out of account. As already noted, the effect on the living egg is very striking: it appears stratified in the direction of action of the centrifugal force; at the central pole is a small gray cap, next to it a broad, clear or hyaline

band, and the distal hemisphere appears intensely yellow, and packed full of yolk-granules. The surface of apposition of the hyaline band and yellow hemisphere is not a plane surface, but decidedly convex towards the hyaline band.

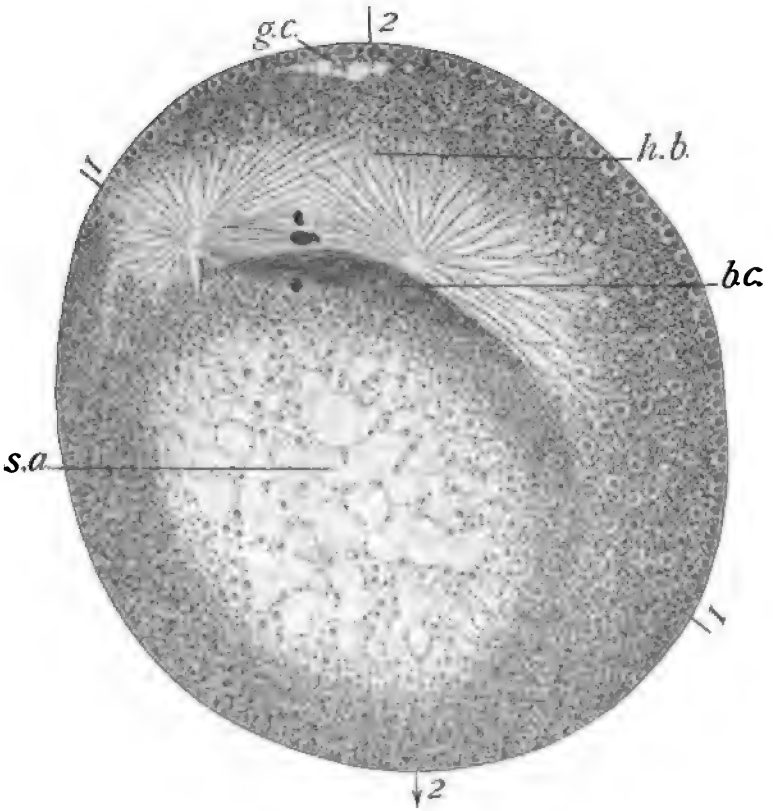

FIG. 6. Section of an egg of *Chætopterus* centrifuged 1,150 revolutions in 31 seconds at a radius of 13 cm. The force was not sufficient to produce a very full segregation of the material of the gray cap. *1–1*, primary axis of the egg; *2–2*, secondary axis. *b.c.*, basophile cap; *g.c.*, gray cap; *h.b.*, hyaline band; *s.a.*, spongy area; stained in thionin and orange *G*.

The gray cap is composed of fat globules, as is demonstrated by staining with osmic acid (Fig. 9); whether or not it includes the residual substance of the germinal vesicle as maintained in my former paper, I am not now prepared to say. In *Nereis*, in place of the gray cap, we have an aggregation of the large oil drops

characteristic of this egg. The hyaline band contains the small basophile granules, and the distal hemisphere the large acidophile granules (yolk-spheres). In sections stained in thionin and orange *G* the hyaline band is bright blue and very finely granular; the distal hemisphere is orange, owing to the affinity of the large yolk granules for this stain. But among the orange spherules are some basophile granules, and with the centrifugal power noted above, some orange spherules may be found in the basophile band. But with a sufficiently high power a practically complete separaration of basophile and acidophile granules may be brought about.

Attention must now be directed to a definite configuration in the distal or yellow hemisphere, viz., a clear, spongy area which extends into the base of the clear band, and which is surrounded distally and laterally by acidophile granules. It is similar to the central spongy area of the normal control eggs with which it must be identified. It contains relatively few granules, which are arranged in a coarse meshwork. The central end of this area is occupied by an aggregation of basophile granules which are so much more densely packed than those in the clear band, with which the special aggregation is continuous, as to contrast strikingly with it in sections; particularly with low powers of the microscope. The form of this aggregation is lunate or biconvex in section, thick in the center and diminishing towards the ends.

The large acidophile granules tend in a distal direction and the smaller basophlle granules in a central direction. If there were no differences in resistance in the ground substance they would pass in straight lines. But the fact is that they do not do so, and this is *prima facie* evidence of differences in resistance of the ground substance in different areas:

The basophile cap in the central boundary of the spongy area is a clear demonstration of this principle: In the first place it is an aggregation of basophile granules; this is proved by the staining reaction, always basic. That the stain is in the granules and not in the ground substance is readily demonstrated in thin sections, and the granules are of the same size as the basic granules of the clear band. In the second place, since such basophile granules tend to move centrally, that is, towards the hyaline band,

under the influence of centrifugal force, this aggregation, which does not exist in the control eggs, must have come from the distal hemisphere. But as they lie at the central end of the spongy area, and are most numerous opposite the center of this area, and diminish towards the sides, it is obvious that they must have come from the interior of this area.

They are in fact a swarm of microsomes moving under the influence of centrifugal force, but delayed on the borderland of the

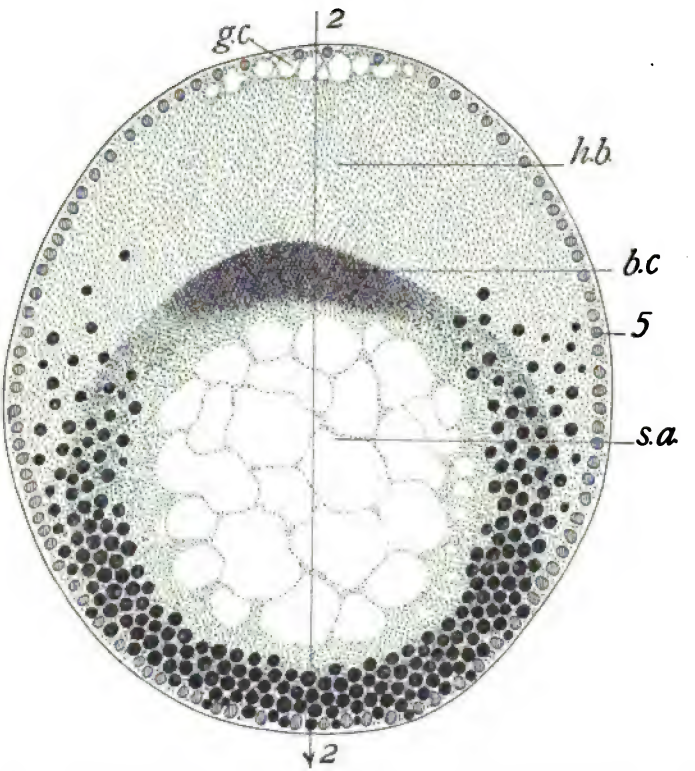

FIG. 7. Section of an egg of *Chætopterus* centrifuged 1,150 revolutions in 31 seconds at a radius of 13 cm. The primary axis of the egg is unknown in this case. *2–2*, secondary axis; *5*, ectoplasmic zone; *b.c.*, basophile cap; *g.c.*, gray cap; *h.b.*, hyaline band; *s.a.*, spongy area; stained in iron hæmatoxylin and orange *G*.

hyaline band (cf. Fig. 7), where they belong by virtue of their specific gravity, by the greater density (or other mode of resistance) of the wall of the spongy area.

This property of the wall of the spongy area also explains the

form of aggregation of the acidophile granules, which tend on this account to pass around and not into it. It also explains the more striking character of the spongy area in the centrifuged and the control eggs (cf. Figs. 6 and 4), because the granules within this area are free to segregate at its ends, but those without are prevented from entering.

Similarly, the persistence of the granules in the ectoplasmic zone indicates that this is another region of greater density of the ground substance. It requires a much greater centrifugal force to dislodge these granules than those lying within the egg, but when they are so dislodged they appear to be the heaviest class of granules within the egg, for they aggregrate at the distal pole (Fig. 9).

The following diagrams (Fig. 8) illustrate both the conception of the ground substance to which I have come as a result of the experiments, and the effect of the centrifugal force on the ground substance itself: The latter consists of four concentric layers, 2, 3, 4 and 5 (Figs. *A* and *C*); of which 5, the ectoplasmic stratum, is relatively dense, 4, the zone containing most of the acidophile granules of the normal egg is relatively fluid, 3, again, is a dense ring-shaped zone, surrounding 2, the central, more fluid spongy area.

The egg is an elastic sphere; it therefore undergoes elongation in the direction of the centrifugal force (Fig. 8, *B* and *D*); this is perfectly obvious immediately after centrifuging. The arrows in the figures to the left indicate the direction of the centrifugal force and the figure to the right in each case represents the result. Now observation shows that the spongy area of centrifuged eggs lies in the distal hemisphere, hence elongation of the ground substance must have taken place on the central side, owing presumably to the tension of the specifically very light substances (*e. g.*, fat globules) found here.

I have employed five or six different degrees of centrifugal force in examining this question and have made a considerable number of experiments with each. The configuration which we have just described varies somewhat with different degrees. With less force the same configuration is found down to 575 revolutions in 17 seconds with a radius of 13 cm. With higher forces

it tends gradually to disappear as is to be expected, for it is obvious that with a sufficient force the granules would be driven through or out of areas of greater density very readily and would segregate solely with reference to their specific gravity. With a

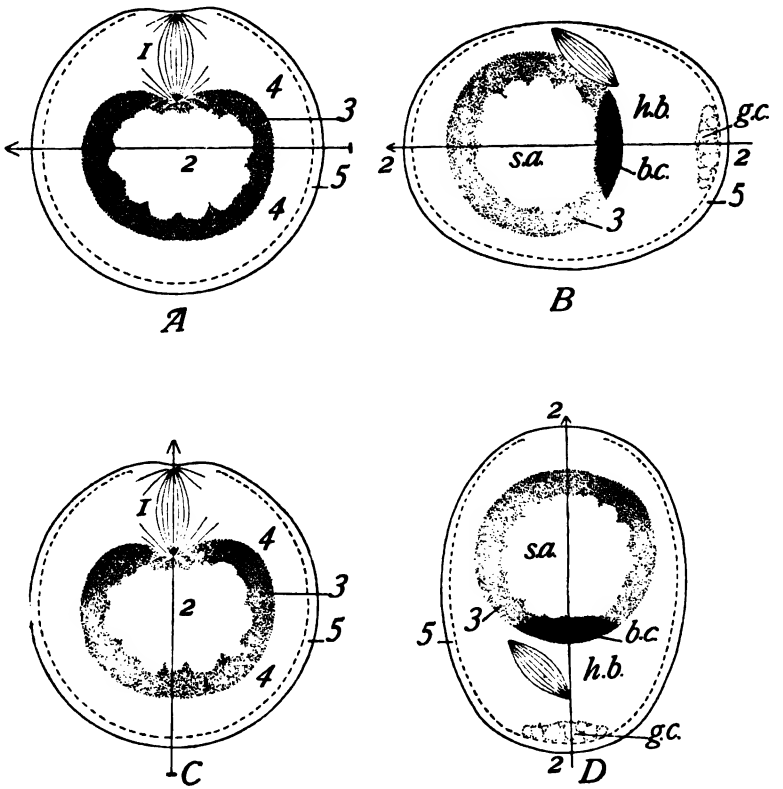

FIG. 8. Diagrams to illustrate structure of the ground substance of the egg of *Chætopterus* as revealed by centrifuging. *A* and *C*, controls: the arrow in each represents the direction of the centrifugal force. *B* and *D*, resultant conditions after centrifuging; *B* when the direction of the centrifugal force is as in *A*, *D* when the direction of the centrifugal force is as in *C*. *1*, spindle area; *2*, spongy area of control eggs; *2-2*, secondary axis of centrifuged eggs; *3*, dense ring-shaped zone in the ground substance; *4*, relatively fluid zone; *5*, ectoplasmic zone. The numbers have about the same significance as in Fig. 4. *b.c.*, basophile cap; *g.c.*, gray cap; *h.b.*, hyaline band; *s.a.*, spongy area of centrifuged eggs.

speed of 7,500 revolutions in one minute at a radius of about 6 centimeters, one can hardly observe any trace of the concentric arrangement (Fig. 9). Abundance of proof can be furnished for

these statements, but there would not be much value in multiplying instances by describing all of the intermediate conditions.

The same configuration is found in all stages of maturation and fertilization up to the first cleavage, at which time the cleavage naturally causes modification. Whether or not it reappears in each cell is a question that I have not examined.

It may be objected that the configuration shown could be produced as readily by mass movements of the protoplasmic areas as by individual granule movements. But such a conception would meet a fatal difficulty in the case of the central spongy area; the basophile cap in the central end (with reference to the centrifuge) of this area could not be produced in any other way than by a granule movement. Moreover, detailed examination of the movements in the spherular zone (Fig. 4, *3*) seem to me to be equally conclusive: If the movements produced by centrifugal force in this area were mass movements of the protoplasm as a whole the conformation of this zone should vary with the direction of the centrifugal force until it reached a final position of equilibrium, for the reason that it would fold in different ways around the central spongy area. The graded series of centrifugal forces employed may be regarded as showing stages in the segregation of the granules of this area. If the movements were mass movements the animal pole extensions of the spherular zone (Fig. 4) must wrap around the central spongy area where the centrifugal force acts at any appreciable angle from the egg-axis. But this is not the case; the proximal pole of the spongy area is never covered by such a fold. With the lowest speed, in directions at any appreciable angle to the egg-axis, one finds that the hyaline zone is dotted with granules of the spherular zone, evidently caught in migration; such granules are rarer with successively higher powers of the centrifuge, until they are practically absent. It is obvious that they pass individually across the hyaline zone.

Another conclusive proof of individual granule movements is shown in Fig. 9. The spindle is in a very high degree resistant to penetration, and it therefore often acts as a block to migrating granules, which pile up against it. The figure illustrates the blocking of fat granules which are for the most part accumu-

lated at the central end of the egg (upper end in the figure). Innumerable cases of this kind are found in the preparations.

Again, as already noted, the ectoplasmic granules tend to keep within the ectoplasmic zone with low powers of the centrifuge.

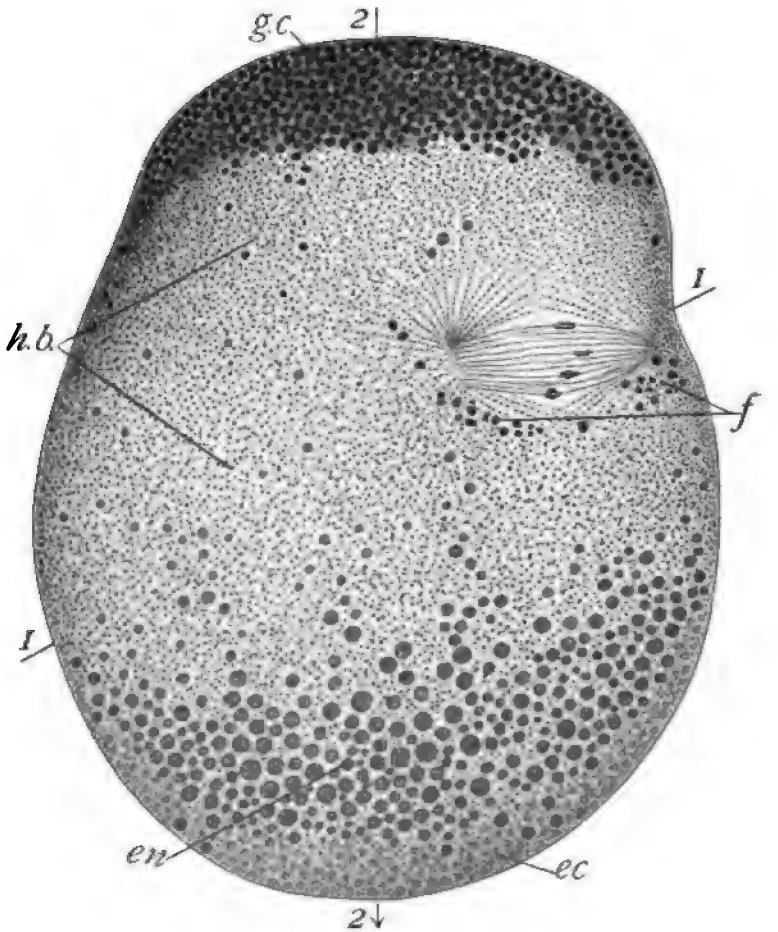

FIG. 9. Section of an egg of *Chætopterus* centrifuged 7,800 revolutions in one minute at a radius of 6 cm. The egg was killed in Flemming's fluid, and the fat granules of the gray cap are stained black. The spindle blocks fat globules passing in a central direction (*f*). *1-1*, primary axis; *2-2*, secondary axis; *ec.*, ectoplasmic spherules; *en.*, endoplasmic spherules in the distal hemisphere; *f.*, fat globules blocked by the spindle, *g.c.*, gray cap; *h.b.*, hyaline band.

But with higher powers they pass to the distal pole of the egg. Nevertheless the ectoplasmic zone may be recognized without

the granules, after fixation with Flemming's fluid, as shown in Fig. 9.

The conclusion of this whole matter is that there is demonstrative evidence of individual movement of all classes of granules under centrifugal power, and no evidence of mass movements of protoplasmic areas. These movements reveal a definite form of concentric organization in the ground substance, which is only indistinctly revealed by the granule arrangement seen in the normal egg.

It does not require much argument to show that the organization of the ground substance is the primary factor that determines the concentric arrangement of the granules. The idea that the organization in the ground substance is secondarily produced by a given arrangement of granules is contrary to the entire series of results contained in this paper.

The granules reach their position and form the normal segregation pattern by "flowing" movements. Unless the entire argument of the present paper is incorrect, the flowing movements are simply granule movements within a firmly organized ground substance, determined by unknown factors involved in the relations of nucleus and granules to each other and to the ground substance.

It is incredible to me that vital organization can be bound up in a flux of all protoplasmic constituents. Vital processes must be bound up with some firm condition of aggregation in the protoplasm.

But how, it may be asked, are polarity and bilaterality connected with the concentric organization of the ground substance revealed by centrifuging? The answer to this question is not at all obvious. These properties are shown by the experiments to be properties of the ground substance; and the experiments further show, as illustrated in Fig. 8, that the ground substance is merely distorted, not essentially altered by centrifugal force, and this furnishes a physical basis for the persistence of polarity and bilaterality in the original directions. But their nature is not in the least revealed by the experiments. The results only furnish a partial answer to the old question, how life and organization can exist in a total flux of materials, by showing that the flux is only partial.

Morgan has made the suggestion (*Science*, August 28, 1908, p. 288) that formative substances other than the visible granules here referred to may possibly be present in the sea urchin's egg, and that such materials are not seriously disturbed by a centrifugal force sufficient to separate the visible substances of the egg; but that if these more fundamental substances are displaced, interference with the normal development would be expected. According to this conception vital organization would still be a function of equilibrium and interaction of diverse substances. The logical possibility of such a conception must be admitted, but it avoids none of the difficulties inherent in the usual hypothesis of organization, and seems to me to offer no possibility for explaining the polarity or bilaterality of parts of an ovum or the phenomena of regulation in general.

The existence of polarity and bilaterality in an optically homogeneous medium, and the persistence of both as to orientation under experimental conditions that seriously modify the quantitative relations of the oriented medium in different regions (as, for instance, when the yolk granules are packed closely into the small cell of the two-celled stage of *Chætopterus*) seem to me to argue for a molecular basis of the fundamental principle of vital organization.

Lehmann's observations on fluid crystals offer interesting analogies that are suggestive in the consideration of such fundamental biological problems. They show that the conception of crystallization as applied to vital organization does not meet with some of the difficulties formerly considered inherent in the analogy. Particularly his observations on the so-called myelin forms where innumerable fluid crystals determine the form and behavior of a single aggregate, suggest most interesting biological analogies, and aid to a certain extent in conceiving a doubly heteropolar condition of aggregation such as is presented by the animal egg.[1]

[1] It is not of course the author's intention in this communication to attempt to establish the validity of the analogy, but only to point out its possible application to the demonstrated organization of the ground substance of the cytoplasm. A good discussion of the entire analogy of crystallization to morphogenesis is contained in Przibram's article "Kristall-Analogien zur Entwicklungsmechanik der Organismen," *Archiv für Entw'mech.*, Bd. XXII., 1906.

The principle laid down here as to the molecular basis of cytoplasmic organization must apply not only to the stages considered but to all stages of development and to all parts of the organism.

The polarity of the egg and of living parts generally has been compared to magnetic polarity. Thus parts of a magnet exhibit polarity in the same direction as the original whole, and the north pole of the part is as true a north pole as the north pole of the whole magnet; the same is generally true of polarity in living things: a piece of *Hydra*, for instance, exhibits the same polarity as the entire animal of which it is a part. But push the comparison a little farther and the analogy appears to break down: a piece from the north end of a magnet has no more northness, so to speak, than a piece of the same size from the south end; on the other hand, an animal piece of an entire egg or an oral piece of an entire animal frequently has considerably more animal or oral properties than a piece from the vegetative or aboral end. The same difficulty of course arises if we use the analogy of crystallization. But it may be that it is due to secondary causes that modify the result without detriment to the validity of either analogy.

In conclusion a few words with reference to the so-called formative stuffs: So far as they are to be identified with the visible substances segregated by the centrifuge, it would appear to be indicated by the experiments that they can play no specific rôle in differentiation, because in centrifuged eggs they may occupy variable positions in the embryo. I believe, however, that the matter requires further investigation. It seems hardly credible that the strictly determinate distribution of these substances in normal ontogeny is meaningless, and that abnormal distribution is a matter of indifference. It is noteworthy that a large proportion of centrifuged eggs die before development has proceeded far, and that there is a distinct tendency towards abnormalities in the surviving eggs. It is natural to correlate these facts with unregulated abnormalities of distribution of granular materials. In any event a more careful study of the facts is needed.

It should be borne in mind that only the factors of polarity and bilaterality, the localizing factors in general, are shown to be functions of the ground substance. The respective rôles of

nucleus and granules are undetermined by these experiments; but their importance in the physiology of development is not open to question.

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KARYOKINETIC FIGURES OF CENTRIFUGED EGGS; AN EXPERIMENTAL TEST OF THE CENTER OF FORCE HYPOTHESIS.

FRANK R. LILLIE.

In the annelid *Chætopterus* the first maturation spindle forms in the unfertilized eggs after they are shed in the sea-water, and develops to the stage of the mesophase (Fig. 1). Here it remains standing without change, it may be for hours, unless the egg be fertilized or otherwise effectively stimulated. The eggs can be obtained at Woods Hole in unlimited amounts for a period of about two months during the summer, and as they may be taken from the worms at any time of day they furnish ideal material. The possibility of experimenting directly on a definite stage of the karyokinetic figure is naturally suggested by the material itself, and I propose to give here an account of the results of experiments with centrifugal force, which have been carried on during four successive seasons, so far as they are related to the karyokinetic figure. A communication of the results was made before the joint session of the Central Branch of the American Society of Zoölogists and Section F (Zoölogy) of the American Association for the Advancement of Science held in Chicago, December, 1907, and an abstract was printed in *Science*, N. S., Volume XXVII., pp. 907–908, June 12, 1908.

The principal advantage of this material, aside from its availability and abundance, is that it offers the rare opportunity of experimenting on a *definite* and *fixed* stage of the karyokinetic figure. At the stage in question the forces concerned in karyokinesis have reached a certain balance which is maintained indefinitely, until the equilibrium is upset by the conditions initiated by entrance of the spermatozoön or some other effective change.

The action of potassium chloride appears to be quite specific in this respect, as was first discovered by Mead ('98), and since described by Loeb ('01) and myself ('02). Such agents set in motion the series of karyokinetic processes, which are then carried through to their completion. But even violent mechanical disturbances, such as are produced by shaking or centrifuging, cause only temporary disturbances, and the equilibrium of the mesophase of karyokinesis is soon reëstablished. The methods

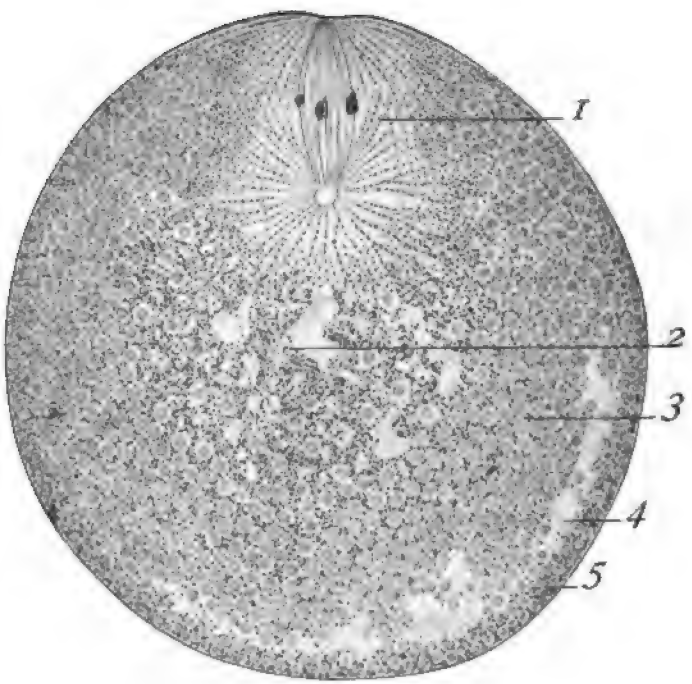


FIG. 1. Section of an egg of *Chætopterus* at the stage used for the experiments. See text for description. 1, karyokinetic figure; 2, central spongy area; 3, inner dense zone; 4, outer spongy area; 5, ectoplasmic layer or outer dense zone.

by which the regulation, or regeneration, of the karyokinetic figure is brought about after centrifuging appear to me to have some bearing on the problem of the nature of the dynamical complex represented by the karyokinetic figure.

Fig. 1 shows the typical condition of the eggs before centrifuging. The animal hemisphere contains a large karyokinetic figure at the mesophase with nine chromosomes in the equatorial

plane. The outer pole of the spindle is practically in contact with the surface of the egg, which is slightly depressed at the pole. The centrosome of the outer pole is smaller than that of the inner pole. The astral rays cross very extensively opposite the equatorial plane; indeed, rays from the outer pole may extend inward beyond the level of the inner pole, and rays of the latter may extend nearly to the surface of the egg. In the spindle itself, there is the usual distinction between mantle fibers attaching the chromosomes to the two poles and central spindle fibers running from pole to pole. The interfibrillar spaces are perfectly clear. The only asymmetrical feature of the spindle is the somewhat smaller size of the outer pole and the difference in arrangement of the rays at the two poles, owing to the proximity of the outer pole to the surface of the egg.

All eggs show great uniformity, but there may be slight differences between eggs of the same lot in the following respects: (1) In some the outer pole is not actually attached to the surface so as to produce a depression, but may be removed from the surface by a distance of about twice the diameter of the outer centrosome. There is thus a variation in the firmness of fixation of the spindle at the animal pole. (2) There is some variation in the intensity, that is, in the length and strength of the astral rays, which appear less well developed in some cases than in others. In all cases, they cross to a considerable extent in the equatorial plane.

The evidence that is to be presented is furnished by the results of centrifuging the eggs at varying speeds and for various times. To form a proper conception of what happens it is absolutely essential to understand the effects of centrifugal force on the protoplasm. In a previous paper I presented an analysis of this subject and showed that the fundamental structure of the egg as represented by the conformation of the ground substance is not essentially modified by the centrifugal force; it is at most somewhat distorted. On the other hand, the granules suspended in the ground substance move through the latter in a central or distal direction, depending on their relative specific gravities, and arrange themselves in three strata: the stratum nearest the axis of the centrifuge is of course of least specific gravity; it consti-

tutes a cap of granules of intermediate size that give a fatty reaction with osmic acid and are soluble in oils. The intermediate stratum, forming a *hyaline band*, contains very minute granules that take the basic stain; they may therefore be called basophile granules. The distal stratum occupies the distal hemisphere of the egg and is composed of larger granules of yellow tinge, usually called yolk granules, which take the acid stain and may be known collectively as acidophile granules. The chromatin and the spindle have the same specific gravity as the basophile granules, as is proved by the fact that they are found in the hyaline band when they move freely.

The movements of the granules, and consequently their segregation, are modified by differences of resistance within the ground substance. The latter has four concentric zones (Fig. 3, *A*), (1) a central more fluid area (Fig. 3, *A*, 2), (2) a dense zone bounding the latter (Fig. 3, *A*, 3), (3) a second more fluid zone specially developed in the region of the spindle (Fig. 3, *A*, 4), and (4) an ectoplasmic zone defective at the outer end of the spindles (Fig. 3, *A*, 5) (see Lillie '09).

All zones contain both basophile and acidophile granules (Fig. 1). Under the action of centrifugal force, therefore, the granules move relatively freely through the more fluid zones and are impeded in their movements in the denser zones. The consequence is that when low centrifugal speeds of short duration are used, there is a tendency for the granules to aggregate in the dense zones. Thus the basophile granules of the central more fluid area aggregate in the central end of the dense zone bounding it (Fig. 2) and the acidophile granules in the distal end. The basophile granules of the outer fluid zone aggregate proximally with reference to the axis of the centrifuge and the acidophile granules aggregate distally, and therefore lie external to the inner dense zone and within its walls, but do not pass through the latter to enter the central more fluid area (Fig. 2). The most important of these aggregations with reference to the interpretation of the karyokinetic figure is the *basophile cap* found in the central end of the inner dense zone (Fig. 2, *b.c*).

The distortion of the ground substance is of the following character: (1) the egg is invariably somewhat elongated in the

line of action of the centrifugal force, owing to the differential force between centripetally and centrifugally moving granules; (2) the principal part of this elongation is born by the massive, external, more fluid zone, which is stretched more on the centrip-

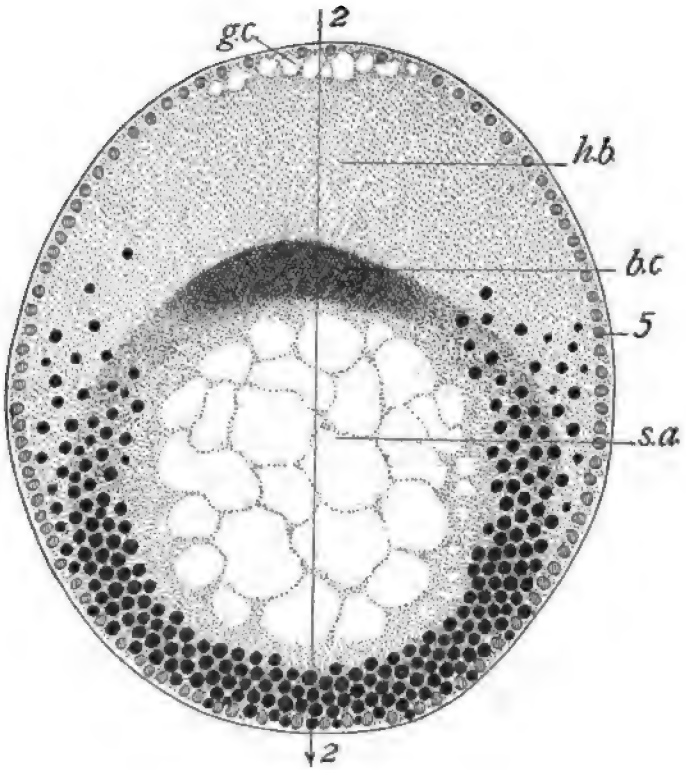


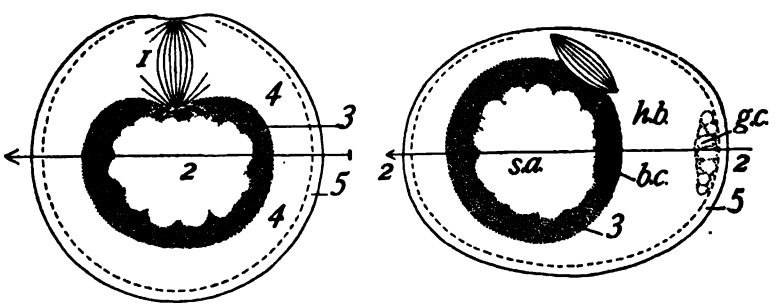
FIG. 2. Section of an egg of *Chætopterus* centrifuged 1,150 revolutions in 31 seconds at a radius of 13 cm. 2–2 indicates the direction of the centrifugal force; 5, ectoplasmic zone; *b.c.*, basophile cap; *g.c.*, gray cap, or fatty granules (dissolved out by the oil used in preparation); *h.b.*, hyaline band; *s.a.*, spongy area. This egg was stained in iron hæmatoxylin and orange G.

etal side, the consequence being that the inner fluid area and its dense bounding zone lie on the centrifugal side of the middle point of the elongated egg (Figs. 3 and 4).

The spindle itself may be moved as a result of centrifuging. The simplest cases are those in which the outer end remains fixed and the inner end is swung from its radial position. This shifting appears to be due to distortion of the ground substance with

the result that the inner dense zone presses in one or another direction on the spindle and displaces its inner end. In other cases the spindle is moved some distance from the surface into the hyaline band; this result is more commonly found with the higher centrifugal powers.

The normal relation of the spindle to the zones of the ground substance is illustrated in Figs. 3, *A*, and 4, *A*. The inner end of the spindle reaches to the inner dense zone and its antipolar rays are embedded in the latter, which is indented by the spindle. If now the centrifugal force acts parallel to the axis of the egg with the animal pole in a central position, the egg elongates in this axis. If the outer end of the spindle remains attached to the surface, the inner end tends to be withdrawn from the inner dense zone, and the basophile cap formed lies outside of the area of



A *B*

FIG. 3. *A*, diagram of zones of the ground substance in the normal egg and the relations of the spindle. *B*, diagram to illustrate the effect of centrifugal force on the ground substance and spindle acting in the direction of the arrow. 1, karyokinetic figure; 2, central spongy area of control egg; 2–2, direction of action of centrifugal force; 3, inner dense zone; 4, outer more fluid zone; 5, ectoplasmic zone. The numbers have essentially the same significance as in Fig. 1; *b.c.*, basophile cap; *g.c.*, gray cap; *h.b.*, hyaline band.

the spindle. If, however, the inner end remains fixed, the outer end is withdrawn from the surface, and the inner end is embedded in the basophile cap.

If the centrifugal force acts at right angles to the axis of the egg, the inner end of the spindle tends to be swung in a proximal direction, (Figs. 3, *A* and *B*) both because the specific gravity of the spindle as a whole carries it in that direction, and also because the contraction of the egg in its own axis brings the dense zone

nearer the animal pole and thus exerts pressure on the spindle. Many cases of this kind are found.

If the animal pole is distal in position in the centrifuge the spindle must be subjected to rather violent assaults: first, because its specific gravity tends to drive it towards the opposite end of the egg; second, because the basophile granules are streaming away from it and the acidophile granules rushing in, and third, because the inner dense zone is approximated to this pole (Fig. 4, *B*). I have not found any eggs with the spindle fixed at the distal pole after centrifuging, and am therefore forced to assume that in all cases in which the centrifugal force acts in this direction the spindle is torn loose from the surface and moves up into the hyaline bnd.

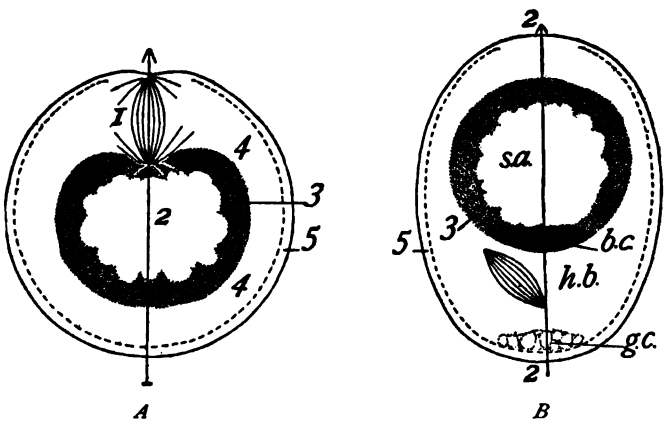


FIG. 4. *A*, diagram of the zones of the ground substance in the normal egg and the relations of the spindle. *B*, diagram to illustrate the effect of centrifugal force on the ground substance and spindle acting in the direction of the spindle. Numbers and letters as in Fig. 3.

Intermediate directions of action of the centrifugal force will affect the protoplasm and the spindle in ways easy to deduce from the above.

If the above analysis of the effects of centrifugal force is correct, or even partially so, we have to distinguish between those cases in which granules are driven out of, or into, the area of the karyokinetic figure, and those cases in which the spindle has moved its position. It is obvious that both conditions may occur simultaneously; indeed, it is probable that this is almost always the case.

In my paper of 1906, I described and illustrated a naked spindle with centrosomes at its ends, but practically no radiations. This spindle was found in an egg that had been centrifuged about 2,000 revolutions in a minute at a radius of 13 cm., killed in picro-sulphuric acid, sectioned, and stained in Delafield's hæmatoxylin. At the time, I was not paying special attention to the question of analysis of the karyokinetic figure, and I regarded the case as typical.

When I repeated the experiments in the summer of 1906, with special reference to the analysis of the karyokinetic figure, I soon found that the condition of a naked spindle after centrifuging was not typical; on the contrary, it is extremely rare and exceptional. Well-developed asters are the rule after centrifuging, though they may exhibit certain asymmetries or other modifications of the normal condition. Either, then, the rays must persist in spite of movements of the spindle and passage of granules through them, or the naked spindle must have formed new radiations.

Presumably the latter process would require time, and it therefore seemed possible that if special pains were taken to fix the eggs in the shortest possible time after centrifuging, one might find at least a considerable proportion of naked spindles. But this also proved a vain expectation. Typically, astral radiations are well developed after centrifuging; and this is the case no matter how quickly the eggs are killed, nor whether the speed be high or low, within the limits of the actual experiments.

I therefore considered the possibility of fixing the eggs during the action of the centrifugal force, but I did not see clearly how to compass this end and so the experiment was never performed.

I. Movements of the Spindle.

Every case in which it can be demonstrated that the spindle has moved through the cytoplasm furnishes a test between mitome and centrosome hypotheses. If the visible rays are organic radii attached to the poles of the spindle (mitome hypothesis), they must be disarranged by each movement of the latter, and movements of the spindle at different angles to its own axis must produce different characteristic modifications of the rays of

the aster. If the spindle moves in the direction of its own axis, for instance, the rays of the forward aster must tend to be bent back about the spindle, and those of the other aster away from the axis. Similarly, there should be definite configurations characteristic of every angle and amplitude of movement. But, if the rays are expressions of forces centered at the poles and are therefore composed of oriented particles (centrosome hypothesis), such disarrangement of the radii would not be expected to result from movements of the spindle, except to the extent involved in the inertia of the substances concerned.

In a considerable number of experiments it was my aim to kill the eggs in the shortest possible time after centrifuging. The tube was removed from the centrifuge as quickly as possible, the water poured off and the killing fluid poured on immediately. Therefore, in two or three seconds after the centrifugal force had ceased to act the eggs were submitted to the action of the killing fluid. Such eggs were sectioned and stained.

The cases that interest us most here are those in which the spindle is found detached from the surface, because it is obvious that such spindles have been moved from their original position: The entire karyokinetic figure in such cases may be perfectly symmetrical if it is found in protoplasm of uniform composition. But differences in the composition of the protoplasm within the area of the karyokinetic figure are correlated with asymmetries of the figure itself which are considered beyond. If the outer pole of the spindle is withdrawn from the surface it is found to possess well-developed antipolar rays, although these were entirely wanting before centrifuging (cf. Fig. 5), and the astral rays are symmetrical around both poles, although "organic radii" would naturally be distorted by such displacement of the "centers of insertion" as is illustrated in Fig. 5. It appears to me that the astral radiations in such cases must be regarded as new. The case is of course analogous to the shifting of sperm asters in fertilization. In the present case, however, we know the time of readjustment to be very short, one minute at the outside, and the initial and reconstructed conditions are before us for comparison (cf. Figs. 1 and 5).

Similar cases are numerous in eggs that have been submitted

to a very strong centrifugal force, 7,500 revolutions in one minute at a radius of 6 cm., because the end effect of such centrifuging is to produce a uniform arrangement of basophile granules in the hyaline band, into which the spindle also is driven. We there-

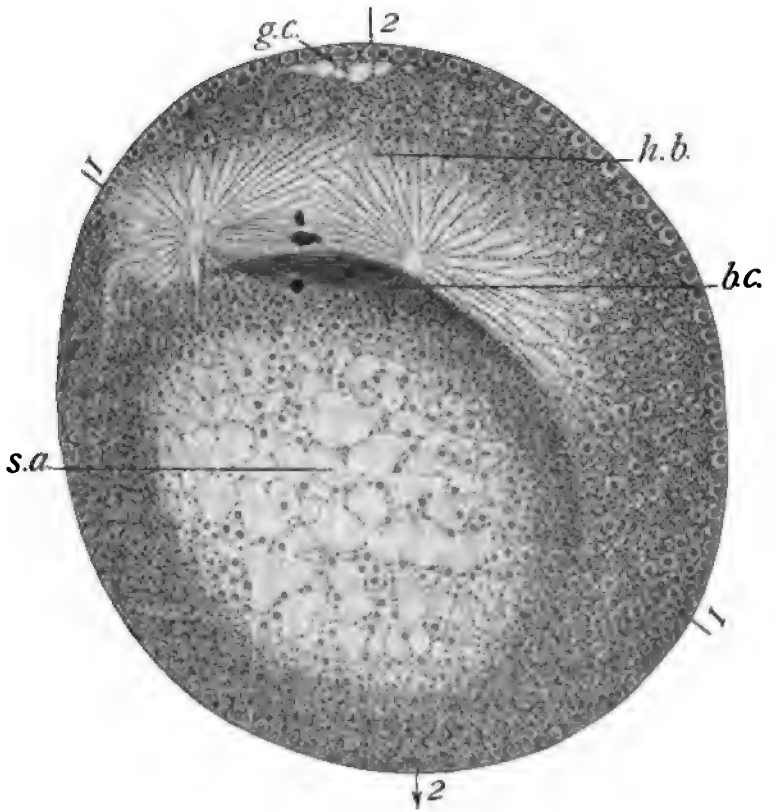


FIG. 5. Section of an egg of *Chætopterus* centrifuged 1,150 revolutions in 31 seconds at a radius of 13 cm. 1–1, primary axis of the egg; 2–2, direction of action of the centrifugal force, secondary axis; *b.c.*, basophile cap; *g.c.*, gray cap; *h.b.*, hyaline band; *s.a.*, spongy area. The egg was stained in thionin and orange G.

fore never find such particomposition spindles as in low powers of the centrifuge (see beyond). The karyokinetic figure is usually symmetrically developed after the strongest centrifuging in spite of the fact that rearrangements of granules are more extensive than with lower centrifugal powers. The reason for this must lie in the uniform character of the protoplasm, which is produced in the hyaline band by strong centrifuging.

A single example of this kind would not be convincing in itself, for it might be maintained that the egg in question deviated originally from the norm of the control eggs. But when such a

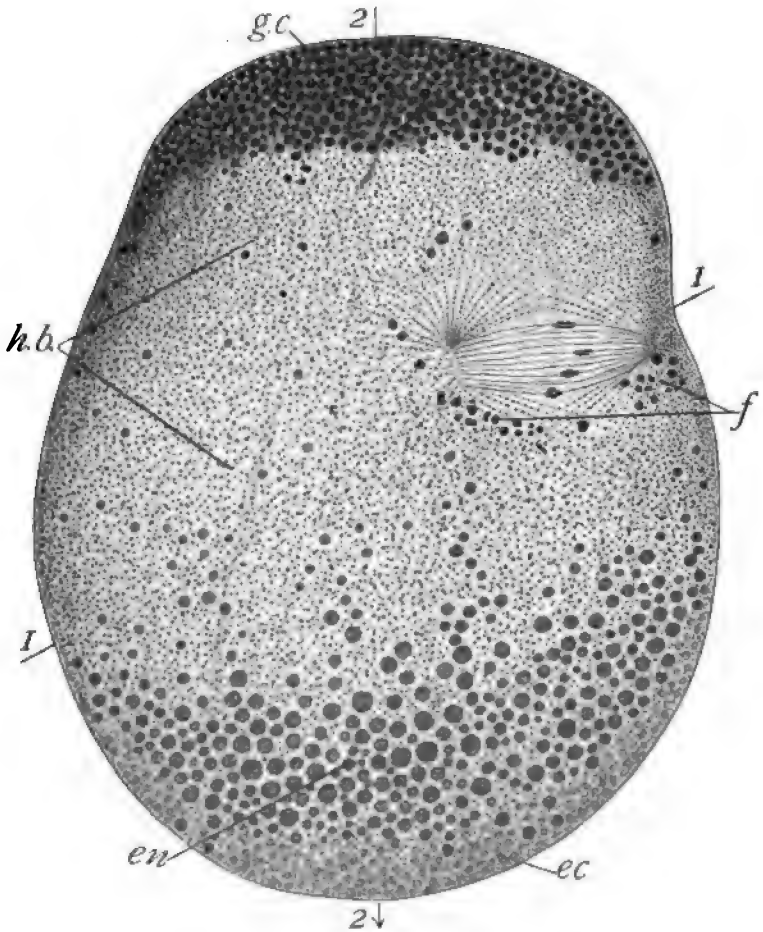


FIG. 6. Section of an egg of *Chætopterus* centrifuged 7,800 revolutions in one minute at a radius of 6 cm. The egg was killed in Flemming's fluid, and the fat granules of the gray cap are stained black. The spindle blocks fat granules passing in a centripetal direction (*f*). 1–1, primary axis of the egg; 2–2, secondary axis; *ec.*, ectoplasmic spherules; *en.*, endoplasmic spherules; *f.*, fat granules blocked by the spindle; *g.c.*, gray cap; *h.b.*, hyaline band.

condition is found to be the type of hundreds of eggs in different experiments with no considerable deviations, as is the case in my

experiments, it becomes a conclusive argument for the re-formation of rays in successive positions of the spindle, a condition that can be explained only on the centrosome hypothesis.

Among the numerous cases we find some in which the antipolar rays are very slightly developed at *both* ends; and various intergrading conditions that seem to indicate stages in the formation of new rays.

In the case of eggs submitted to low centrifugal force an occasional karyokinetic figure is found with distorted astral rays. But such a condition must occur as a transition stage in every case of spindle movement on the centrosome hypothesis, because of the mere viscosity of the medium and the inertia of the oriented particles. The occurrence of such conditions is therefore to be expected, and does not furnish any argument against the center of force hypothesis.

2. Movements of Granules into the Area of the Karyokinetic Figure.

The three classes of granules, basophile, acidophile, and fat, may be moved into the area of the karyokinetic figure. As a general proposition we may say that fatty or acidophile granules driven into the area of an aster tend to destroy the radiations and are not themselves arranged in lines. If forced into the spindle itself, they tend to disarrange its fibers. On the other hand, basophile granules that are driven into the area of the asters or spindle do not, apparently, destroy the existing rays or fibers, and are themselves arranged conformably to the lines of the figure.

A. We need not dwell long on the statement concerning fatty and acidophile granules. It is sufficiently supported by Figs. 5 and 6 as regards the asters. No traces of rays are left in invaded areas of the asters except that the scanty basophile granules between the fatty or acidophile granules may show a slight radiate tendency centered at the poles of the spindle. There are no detached rays, no traces of broken down threads; the peripheral ends of rays of invaded areas always disappear absolutely.

Serious disarrangement of the fibers of the spindle is found if fatty or acidophile granules are driven into it. Such cases are

relatively rare, but the disarrangement may be much more considerable than in the case illustrated (Fig. 7). The ends of the spindle may be much spread out and the chromosomes may be turned around at right angles to their normal orientation.

B. The effect of the introduction of basophile granules into the area of the karyokinetic figure is, however, entirely different. They cause no disarrangement, but appear to become oriented like the rays of the aster, or the fibers of the spindle. The evi-

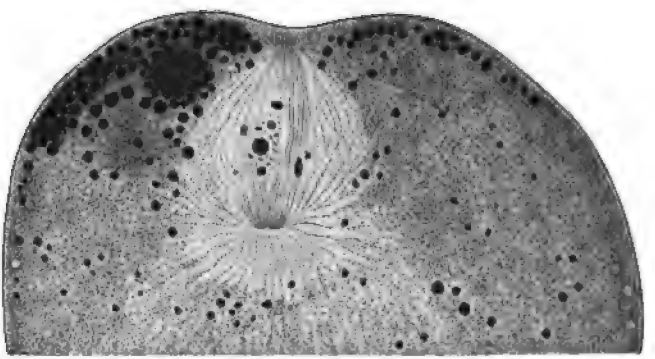


FIG. 7. Section of an egg of *Chætopterus* centrifuged 2,300 revolutions in 70 seconds at a radius of 13 cm. Killed in Flemming's fluid. The figure represents the centripetal half of the egg. Fat granules have entered the spindle and destroyed part of its structure.

dence for this conclusion is based on the behavior of the dense aggregation of the basophile granules found in the central end of the inner dense zone after the action of low centrifugal powers, *i. e.*, of the basophile cap (Figs. 2, 3*B*, and 4*B*).

The heavy stain of the basophile cap lies entirely in the granules, not at all in the ground substance. It is true that the ground substance between the granules appears darker in the basophile cap than elsewhere, but this is an effect due to the light passing through basophile granules at a lower focus. In thin sections it is not evident at all. The conclusion is really proved by the fact that such an effect is seen only in the basophile cap, and that this may lie in any part of the wall of the dense zone, depending on the direction of action of the centrifugal force.

1. Let us note first that the granules that compose the basophile cap have a uniform distribution when remote from the

spindle (Fig. 2). What happens when they are driven into the area of the karyokinetic figure? A few examples will illustrate:

The egg shown in Fig. 8 was allowed to stand in sea-water about 35 minutes, then centrifuged 1,150 revolutions in 31 seconds at a radius of 13 cm. and killed as rapidly as possible in picro-acetic acid. It was one of a large number. Sections were cut 6 μ thick and stained in iron hæmatoxylin and orange G.

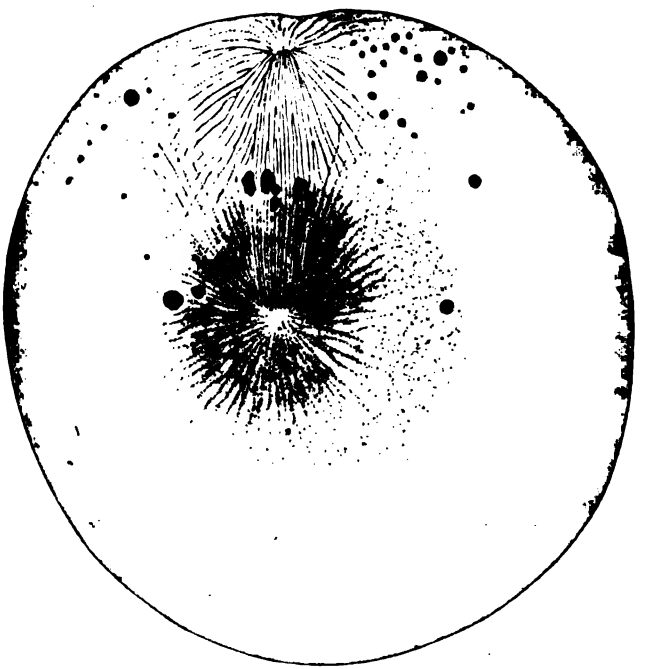


FIG. 8. Section of an egg of *Chætopterus* centrifuged 1,150 revolutions in 31 seconds at a radius of 13 cm. Half of the karyokinetic figure lies in the basophile cap. See text for further description.

All the sections of this egg are present on the slide. The figure represents a single section which includes practically the entire spindle and all nine chromosomes. The plane of the section was nearly transverse to the axis of stratification and is approximately tangential to the basophile cap; but on the right side the section passes nearer to the distal pole than on the left side and therefore takes in a good deal of the distal acidophile stratum on this side, while on the left side the section is confined to the

hyaline band. The granules of the ectoplasmic layer are apparently unaffected by the centrifugal force. The karyokinetic figure has been moved little, if at all, as is proved by the fact that there is an indentation opposite the outer pole, and by the relation of the ectoplasmic layer which exhibits the characteristic defect around the outer pole of the spindle, which I described in my first paper.

That portion of the karyokinetic figure lying within the basophile cap partakes of its character, and is as sharply differentiated from the remainder of the figure as the basophile cap itself is differentiated from the protoplasm of the hyaline band. As to the nature of these differences: (1) the stain is much darker within the basophile cap; (2) the rays of the aster are more

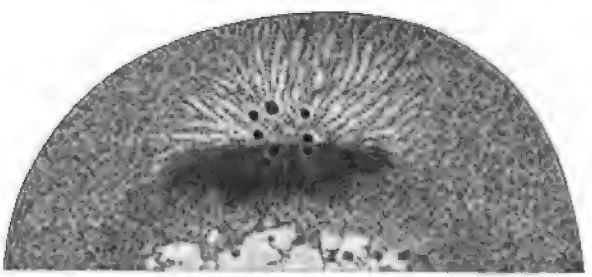


FIG. 9. Transverse section of parti-composition spindle. From section of an egg of *Chætopterus* centrifuged 1,150 revolutions in 31 seconds at a radius of 13 cm. Half of the section of the spindle lies in the basophile cap. See text for further description.

numerous than in the control (Fig. 1) and also more granular; they are in fact obviously made up of linear arrangements of basophile granules; (3) the spindle itself is more darkly stained within the basophile cap than outside of it; this pertains to the fibers themselves, but I would not venture to say that the fibers are more numerous.

Where a fiber either of the spindle or of the aster crosses the boundary between the basophile cap and the hyaline zone its character changes. Rays of the aster never extend into a compact mass of acidophile granules, hence the present figure is less developed on the right side than on the left.

Fig. 5 represents a spindle placed tangentially to the basophile cap. The contour of the spindle is perfectly regular, but its

composition is heterogeneous. For the rest, exactly the same principles apply as in Fig. 8, except that astral rays are not very numerous in the basophile cap; this is due to the fact that the section is near the margin of the cap and contains therefore a large proportion of acidophile granules.

Fig. 9 finally is a transverse section across a spindle lying partly in and partly out of a very dense basophile cap. It is obvious that the spindle area has been invaded on one side by the basophile granules. These are in fact so numerous and are stained so deeply that they almost conceal the chromosomes embedded in them. The concentration of the granules within the spindle area is thus extremely different on the two sides, but on each side it is precisely the same as in the neighboring cytoplasm.

These cases are typical of a large number in my preparations. It is obvious that there is a great difference in the fatty and acidophile granules on the one hand and the basophile granules on the other, with reference to their effect on the karyokinetic figure. The former efface any part of the figure which they occupy, the latter are arranged conformably to the lines of the figure. This difference is not conceivably a mere question of size, it is rather a question of specific behavior. The basophile granules behave as though they were within effective range of centers of force to which they are permeable; the fatty and acidophile granules on the other hand behave as impermeable particles would behave in such a field.

It is worth while to examine this idea more carefully because the center of force hypothesis alone can render account of the results of these experiments. Theoretically, on a center of force hypothesis the following results might be expected: (1) That the number of radiations from the center would be a factor of the density of aggregation of the more permeable particles; if the number of permeable particles within the effective area were greatly increased the number of rays should therefore become greater. (2) The number of oriented particles in the same length of any ray should also be a factor of the density of aggregation of the permeable particles.

We would therefore expect on the center of force hypothesis

of the karyokinetic figure that, if the basophile granules are permeable to the force, (1) they would be oriented along the lines of force when driven into the area of the karyokinetic figure; (2) that the number of rays in an unusually dense aggregation of basophile particles would be greater than usual and (3) that the number of oriented particles in each ray in such cases would be greater than usual.

These appear to be the conditions found in my experiments. With reference to the first condition, there can be no question that the basophile granules arrange themselves conformably to the lines of the karyokinetic figure, as already noted. With reference to the number of astral rays one obtains a strong subjective impression that they are more numerous in the basophile cap than in the asters of control eggs, and I have attempted to confirm this by actual counts. To do this it is necessary to divide the aster into sectors for comparison, for the reason that asters lying entirely within the basophile cap do not occur, owing to the form of the basophile cap. I have therefore made comparisons between sectors of 90° of the normal aster and asters of the basophile cap. One has further to restrict the count by limiting it to a single focus of the microscope. Under these conditions I found an average of 8 rays to the 90° sector of the normal aster (eighteen counts) and 12.5 in 90° sectors of asters in the basophile cap (six counts). The difference seems too great to be accounted for by error. The counts confirm the impression that one receives by mere comparison. I do not think that such increase of the number of rays in a sector of the aster could be explained by compression of preëxisting rays from other sectors of the same aster, for they are quite uniformly spaced and are as straight and regular as the normal rays.

As regards the third condition: rays within the basophile cap stain more strongly than those without; but as the stain is held by the granules, this would be evidence for a greater number of granules in the rays. A ray that passes out of the basophile cap loses suddenly in intensity of staining (Fig. 8).

The spindle is decidedly denser than the surrounding protoplasm; this has been noted by several investigators (cf. Foot and Strobell (p. 221), and McClendon). The same thing is

shown by some of the phenomena of centrifuged eggs; when, for instance, the direction of centrifugal force is at right angles to the axis of the spindle, granules must stream by it. Now if the density of the spindle area be no more than that of the surrounding ground substance and if there be no repellent force from the spindle, such granules would pass through the spindle readily. But on the contrary they are blocked by the spindle, and heap up against it. Fig. 6 illustrates this condition. The accumulation of granules on the distal side of the spindle is fatty in character, at least the granules are blackened by Flemming's fluid which was used for killing, and they are the same in character as those accumulated at the proximal pole. A pathway of such granules runs from the pole of the spindle towards the proximal accumulation, rendering the interpretation certain. We can readily believe, therefore, that the relatively dense spindle moves as a unit through the protoplasm. If, then, it be thrown into the basophile cap, it will naturally tend to maintain its character; many such cases are in fact found. In other cases, the part of the spindle within the basophile cap is partly changed, partly unchanged; but in very many cases the part within the cap is completely changed, so that the staining reaction of the spindle agrees with that of the cap.

Whether we consider the movements of the karyokinetic figure produced within the egg by centrifuging, or whether we consider the results of driving granules into the area of the karyokinetic figure, the general conclusion that the poles of the spindles are centers of force appears to me to be inevitable; no system of antagonistic fibrillæ could behave in such a way. The difficulties that the centrosome hypothesis has to meet are well known, and their discussion does not enter within the scope of this contribution, which is in a sense a by-product of another problem. I shall therefore be satisfied merely to present the evidence and to indicate the direction in which it appears to point.

In the development of the karyokinetic figure, the rays or fibers appear to grow out of the centers up to the time of the mesophase at least, and this must be due to increasing power of the centers. In a magnetic model on the other hand the orientation of particles along the lines of force takes place simultane-

ously by segregation along their entire course (Hartog, '05). Now my experiments give conditions quite similar to magnetic models in this respect, for the reason that the centers displaced at the mesophase are in the condition of maximum force.

The fact that the experiments deal only with this stage of the karyokinetic figure limits the results in ways that I fully realize. But this is more than compensated by the advantage of knowing the precise stage of the karyokinetic figure with which the operations deal. The attempts to obtain comparable results at other stages have been unsatisfactory, because any other stage is in motion and the control eggs vary so much that interpretation of results becomes very uncertain.

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THE THEORY OF INDIVIDUAL DEVELOPMENT[1]

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ORGANIC development presents two aspects: that of the individual and that of the race, ontogeny and phylogeny (evolution). These are not two separate and distinct series of phenomena; on the one hand, the individual development is to a certain extent a record of the past history of the race, and the promise of future racial development; on the other hand, evolution is not a series of completed individuals but a series of individual life, histories; for the only road from one generation to the next is by way of a complete life history. Individual development is, therefore, not something distinct from evolution; it is a part of the process of evolution itself; the development of the individual is a chapter in the history of the race.

The development of the individual may be pictured as a steadily broadening stream that takes its source in the fertilized ovum and flows on until death. In this analogy the individual would be represented as a cross-section of the stream at whatever stage we were examining. Though such an analogy limps, inasmuch as individual development is never before us as a unit, as a stream may be conceived to be, and can indeed be said to exist only as the successive cross-sections (its past having disappeared and its future yet unborn), nevertheless, it represents very well the steady, unbroken progress of development from the ovum to old age. There may be crises in the development of the individual, as, for instance, when the chick leaves the egg or the pullet lays its first egg, but there are no breaks in its continuity. Successive generations may be pictured as new streams, each taking its source from a particle—a germ cell—from some cross-section of the preceding generation; and evolution may be represented by placing the new source at a different level than the original. For evolution studies we compare cross-sections of different developmental streams (generations) at comparable distances from the sources, and for evolutionary explanation we must examine the entire series of processes involved in the origin of the new source and in the conditions and inherent character of the new developmental stream.

We can not be said to have actual experience of any other form of development than individual development; evolution or racial de-

[1] One of the series of Darwin Anniversary addresses given under the auspices of the Biological Club of the University of Chicago, February 1 to March 18, 1909.

velopment is an inference from innumerable facts and series of phenomena, all of which are bound up together and rendered intelligible by the theory of common descent. We therefore find that the founders of theories of evolution turn to individual development as the court of last resort, as the place where evolution may be detected in actual process. For here is found the link that binds successive generations, here variations arise, whether they be mutations or of the ordinary fluctuating kind, whether they be germinal or acquired; here in the individual life history the Lamarckian must look for the reflection of the experiences of the individual back upon the germ; here the adherents of orthogenesis must find their crucial evidence.

In his theory of natural selection Darwin accepted as given the data of individual development. But he saw clearly that the fundamental phenomena of heredity and variation had their seat in the individual development, and he experienced the need of framing a conception that would bind together the phenomena of hybridization, the various forms of variation, atavism, telegony, regeneration, inheritance of acquired characters and the like; and in his volumes on "Animals and Plants under Domestication" he framed the provisional hypothesis of *pangenesis* to include them all. I shall not attempt to present the details of this theory, but I may be permitted to say that, as a matter of logical arrangement of the assumed data, under the circumstances of existing biological conceptions and of the state of knowledge of the time, the theory was well worthy of its illustrious founder. In its way, it was as original as the theory of natural selection, though some of its fundamental ideas had certainly been anticipated by previous writers.

Nor shall I attempt a critical estimate of the value of the theory in the history of science; but I may be permitted to call attention to certain features. In the first place, the theory was overburdened with certain unnecessary conceptions such as inheritance of acquired characters, atavism and telegony. The elimination of these conceptions immensely simplifies the theory of individual development. In the second place, it rested upon a fundamental conception, that of representative particles, which amounts to a denial of the reality of individual development. And in the third place, it assumed certain biological processes—the existence of specific vital particles of ultramicroscopic dimensions, their radiation from parent cells, and their aggregation in other specific cells in a definite architectural pattern—for which there is not only entire absence of evidence, but which are wholly inconsistent with the known facts of cellular physiology. For these reasons the theory had only provisional importance, as indeed Darwin recognized in naming it the provisional hypothesis of pangenesis.

The determinant hypothesis of Weismann, contained in his theory

of the germ-plasm, includes the assumptions of the pangenesis hypothesis, with those eliminated that were made necessary by the conception of the inheritance of acquired characters. For Weismann's gemmules, or determinants, the assumption of somatic origin was unnecessary, and thus, as Professor Whitman states, the entire centripetal migration of Darwin's theory was eliminated, but the entire centrifugal process was retained. The origin of every character of the individual was explained in the Weismannian theory, as in the Darwinian theory, by the unfolding (it can not be called development) of representative particles. Nevertheless, the theory of the germ-plasm played an important rôle in the development of biological knowledge, for it framed a set of ideas in a manner sufficiently logical and definite to serve as veritable working hypotheses or bases of attack. The immense effect of Weismann's writings on the theory of individual development should not be underestimated.

Physiology of Development

The theories of individual development that we have mentioned bear all the marks of provisional or formal hypotheses. Although extremely ingenious and logical, they are based only in small part on analysis of the actual processes and they offer no real explanation of the phenomena themselves; for they really include all the elemental phenomena and merely sum them up; they are definitions that include the matter to be defined; they amount to a denial of the reality of individual development as truly as did the preformation theories of the eighteenth century.

As a series of processes occurring in nature and accessible to experience, the development of the individual is capable of resolution into simpler biological processes, and these presumably into physico-chemical events in the usual sense. All attempts to make such analyses come under the head of Physiology of Development; and this plan of attack on the problems of individual development, known in Germany as developmental mechanics, is one of the most actively pursued lines of biological investigation at the present time. Physiology of Development deals primarily with specific problems, and the results constitute a critical basis for the appreciation of general theories of both individual and racial development. We shall examine some results and principles of these studies, and consider their application to some theories of heredity and evolution.

1. *Embryonic Primordia and the Law of Genetic Restriction.*—In the course of development the most general features of organization arise first, and those that are successively less general in the order of their specialization. Thus the directions of symmetry of the future organism—the oral and aboral surfaces, right and left sides, anterior

and posterior ends—are the earliest recognizable features of organization of bilateral animals, and they appear while the germ is still unicellular. The distinction between outer, intermediate and internal organs next makes its appearance, each at first as a single tissue. The outer tissue then separates into an epidermal and a nervous tissue, the inner tissue into the intestinal and yolk-sac epithelium, the middle tissue into muscle-forming tissue, connective tissue, skeleton-forming tissue, blood-forming tissue, excretory tissue, peritoneal tissue, etc.

For every structure, therefore, there is a period of emergence from something more general. The earliest discernible germ of any part or organ may be called its *primordium*. In this sense the ovum is the primordium of the individual, the primitive outer tissue the primordium of all structures of the skin and nervous system, the primitive inner layer of the intestine and all structures connected with it, etc. Primordia are, therefore, of all grades, and each arises from a primordium of a higher grade of generality.

The emergence of a primordium involves a limitation in two directions: (1) it is itself limited in a positive fashion by being restricted to a definite line of differentiation more special than the primordium from which it sprang, and (2) the latter is limited in a negative way by losing the capacity for producing another primordium of exactly the same sort. The advance of differentiation sets a limit, in the manners indicated, to subsequent differentiation, a principle that has been designated by Minot the law of genetic restriction. This in a merely descriptive way is one of the general laws of individual development, and in it is involved the explanation of many important data in the fields of physiology and pathology.

But, though primordia are thus restricted, they nevertheless have the very important property of subdivision, in many cases at least, each part retaining the qualities of the whole. Thus, for instance, in some animals two or several complete embryos may arise from parts of one ovum. Similarly, two or more limbs may be produced in some forms by subdividing a limb bud. Thus frogs with six hind legs have been produced by Gustav Tornier by the simple process of dividing the primordia of the hind legs with a snip of the scissors, in which case he found that on each side one part of the primordium produced a complete pair of legs and the other the normal leg of that side. This capacity for subdivision of primordia explains large classes of pathological facts—at the same time it furnishes a problem to the student of the physiology of development which has proved a serious stumbling block.

2. *Principle of Organization.*—I have already indicated the existence of direction and localization in the primordial germ of the individual, the unsegmented ovum; the ovum, as we say, is polarized,

and, not only so, but, in bilateral animals, it is bilaterally symmetrical. This is not usually indicated in the form of the ovum, which is typically spherical, but in the disposition and developmental value of its parts. Here we have one of the most fundamental and least comprehended facts in embryology. It has, moreover, been shown that this property of direction and localization resides in the homogeneous, transparent, semifluid matrix that suspends all the visible particles of the protoplasm of the egg. It is probable that primordia of all grades possess similar properties, and, if this is so, we have a principle that goes far to explain the orderly localization of processes in morphogenesis.

This principle is not farther analyzable at present; but, as it may be found intact in parts of primordia no less than in the whole, it probably rests on a molecular basis. The most ready analogy in simpler phenomena is that of crystallization. The study of fluid crystals has furnished us examples of inorganic molecular aggregates in which direction and localization are given in the whole and also reappear rapidly in the parts when the whole is subdivided.

3. *The Rôle of Cell-division in Development.*—The individual organism begins as a single cell, from which all cells of the developed organism trace their lineage by the process of cell-division. This has been regarded as one of the most fundamental factors of the individual development in the theories of Weismann, Hertwig and others. But important as the process of cell-division undoubtably is in development, I believe that it is impossible to ascribe to it in principle more than an indirect effect: Considerable complexity of development is possible among Protozoa, whose body is unicellular, and some ova may carry out under experimental conditions a considerable part of the early development without a single cell-division. Moreover, the same kind of differentiated structure may be composed of one cell or of many, or of variable numbers of cells.

The physiological value of cell-division is no different in principle in developing than in functioning tissues (using these terms in the usual sense). The general law of relative reduction of surface in proportion to increasing mass imposes a size limit on cells, which can be regulated only by cell-division; an internal principle of regulation of cell-size has also been stated by R. Hertwig and Boveri, viz., a certain relationship characteristic of each species between the amounts of nuclear and cytoplasmic matters, so that increase of initial volume of the former involves increase of the latter, and *vice versa*. Corresponding to these principles, we find that individuals of different sizes of the same species vary not in the size, but in the number of the cells; and this is regulated by variation in the number of cell-divisions in different individuals.

Cell-division must necessarily, therefore, have an immense func-

tional significance in development, owing to the principle of relation of functional area to mass. It has also another very important function as an isolating factor. The localizations that arise, owing to the organization process of which we have spoken, are rendered relatively stable and permanent by the formation of cell-walls. Thus the elements of the mosaic are isolated, and each isolated part has the opportunity to grow into a new mosaic. Cell-division is thus an important factor in progressive differentiation, not as a cause, but as a means.

4. *Environment.*—Environment must be conceived in a somewhat broader sense than usual in considering the individual development. The developing embryo has an environment in the usual sense, consisting of all those external conditions that surround it, some of which enter into its development. But in addition to this *extra-organic* environment there is an *intra-organic* one; the developing embryo is not merely a unit on which an extra-organic environment operates, but it is a living mosaic, each element of which may conceivably enter into the development of any other in the sense of being a factor in the process. Each part of the embryo, therefore, has an intra-organic environment consisting of all the other parts, some of which constitute relatively immediate environmental factors, others relatively remote ones.

To illustrate: nerves arise in the embryo from certain centers and grow out in the embryonic tissues, much as roots grow out in the soil; the muscles arise separately and the nerves grow to them and make the proper connections. Is this due to an innate tendency of each nerve to grow in particular paths and branch according to definite laws, or, on the other hand, is it due to a directive stimulus exerted on the growing nerve by developing muscle tissue? The answer can be given only by a suitable experiment: If an abnormal innervation area were brought into the field of growth of a developing nerve, would the nerve entering the abnormal area follow its normal mode of branching, or the one characteristic of the normal nerves of the transposed area? To be specific: the bud of a leg of a tadpole that has as yet no nerves may be transplanted to any region of the body (Braus and Harrison), and it develops as a leg; but it receives its innervation from the nerves of the region to which it has been transplanted, and the mode of branching of the nerve is that of the leg nerves. We may generalize this statement by saying that any nerve may be made to depart from its normal mode of branching and to branch like leg nerves, by bringing a leg bud into its innervation area at the time that the nerve is still growing.

It will be seen that if this is generally true, the constancy of distribution of peripheral nerves is not due to the transmission of nerve-branching determinants from generation to generation, but is a function of the intra-organic environment in each generation.

The case of the determination of nerve-branching by intra-organic relations does not by any means stand alone. The same principle undoubtedly holds for the development of the blood-vessels, which grow along paths determined by the arrangement of organs and tissues and not according to a predetermined law given in the blood-vessels themselves. Color patterns have been shown in some cases to be determined by intra-organic variable relations, as in Loeb's experiments on the determination of the color pattern of the yolk-sac of a fish, which he demonstrated to be due to the positive attraction of the circulating blood for migratory cells that bear pigment. The development of any color pattern was therefore dependent upon blood-circulation, and the form of the pattern upon the pattern of the blood-vessels. The primordia of the eye or the ear transplanted to strange locations in the embryo induce formations in surrounding tissues that are strange to them and characteristic of the normal eye and ear environment. The origin and growth of motor nerve cells has been shown in my laboratory by Miss Shorey to be dependent in the chick on normal muscle development; so that the anatomy of the central nervous system, no less than the peripheral system, is dependent to some extent on the environment. Regeneration of lost parts is dependent for its completion to some degree on innervation, and the normal development of muscle tissue beyond a certain stage is likewise so dependent. These examples might be increased by others, which, taken together, would show that an immense part of what we call inheritance is inheritance of environment only, that is, repetition of similar developmental processes under similar conditions. The bearing of all this on the doctrine of determinants, that characters of the adult are represented by germs of a lesser order in the germ of the entire organism, is obvious.

Many of the problems of heredity, so-called, are not capable at present of such resolution. We may note some instances of this kind and then attempt to analyze the whole matter briefly. The example cited of transplantation of a leg-bud is of this kind: the transplanted leg-bud does not develop into an arm if it be transplanted to the region of the arm, but into a right or left leg, as the case may be, and this is true no matter how early the stage at which the transplantation may be made. It is not possible to change the specificity of such a primordium by any means yet employed. Moreover, there are many other experiments which show that the primordia of a great many structures are definitely specified even before they can be detected by any method of pure observation. Thus if a portion of the medullary plate of a frog embryo be cut out so as to include in the cut part the region that would form an eye in the course of time, and if then this piece be replaced inverted, it is found that the subsequent development of this area is inverted, not restored to the normal, although no trace of organs

was present at the time of the operation (Spemann). In this case, then, the eye appears in an abnormal position.

Correlative Differentiation.—We have cited a series of cases that illustrate two apparently contradictory principles known as the principles of correlative differentiation and of self-differentiation. The part that these play in embryonic development should be analyzed. The data of correlative differentiation may be placed in two categories, one of *behavior* and one of *metabolic relations*. Considering these separately:

Behavior.—Any case of behavior involves a stimulus, and a response; these imply irritability and reaction capacity. To take a simple case, for instance, the contraction of a muscle, the stimulus may be of a variety of kinds, nervous, chemical, electrical, thermic, mechanical; in any case the response is contraction. The nature of the response is given in the system and is limited by its reaction capacity. The muscle cell does not contract for one kind of stimulus and secrete in response to another.

This principle is elementary in physiology and psychology and it must apply also in the physiology of development. It appears to me that it has not been sufficiently borne in mind by students of the subject. Herbst, for instance, divides developmental stimuli into directive, trophic and formative. The first kind of stimulus determines the direction of growth or migration, and so plays an important part in development, a really great part illustrated in two of the cases cited, viz., the mode of branching of nerves, and the direction of migration of wandering cells. Trophic stimuli are those that affect the rate or amount of growth without altering its specific character.

The conception of formative stimuli implies, if it has any meaning whatever, that the nature of a developmental process is determined by the nature of a stimulus. A case often cited is as follows: the two most fundamental parts of the eye, lens and retina, develop from two entirely distinct primordia, the retina from the embryonic brain and the lens from the epidermis. The retina first grows out from the wall of the brain and reaches the epidermis to which it becomes fused. The latter then produces a lens. Now it was shown for some amphibia, that, if the retina fails to reach the epidermis, no lens forms; therefore, it was argued that the production of the lens is due to a formative stimulus exercised by the retina on the epidermis. But in some other cases the lens forms even if the retina be absent; which does not prove that it arises without stimulus, only that this *specific* stimulus is not needed. And the fact that transplanted optic vesicles stimulate lens formation in strange localities from the epidermis merely shows that this form of reaction of embryonic epidermis is widespread at this stage of development.

The instance is valuable as proving that stimuli are important in

development, but useless as an example of a formative stimulus. Morphogenetic behavior, like behavior in other fields, is not a function of the stimulus as to its specificity, but it is prescribed and limited by the reaction capacity of the system. One example is as good as many. We shall not find this principle contradicted by any of the known data of the physiology of development.[2]

Metabolic Relations.—When we consider to what an extent the nature of every biological character is given in its chemical composition, it can be readily understood that, to some authors, physiological chemistry should seem the complete basis of heredity. The characters of every tissue of the body are absolutely dependent on their chemical composition, and even slight variations in chemical composition may completely alter function, appearance or form. For such a statement examples are entirely unnecessary.

The development of characters in the individual is dependent upon the occurrence of definite chemical reactions, upon their rate and upon their degree of completion. It has been shown that the law of acceleration of embryonic development in correspondence with rise of temperature is the same in principle as the law of acceleration of chemical reactions by temperature increase. Numerous experiments have been made on the character of development in the absence of one or other or combinations of the elements normal to protoplasm, with the aim of determining their rôle in development. Herbst, for instance, has made a series of experiments on the development of larvæ of the sea urchin in artificial sea waters, in the composition of which definite elements are wanting. He shows, for instance, that in sea water made up without calcium the skeleton fails to develop, and that the form of the larva resulting is profoundly modified from the normal. In other experiments the potassium or sulphur, or iron, etc., is omitted from the solution, and the effect on the development noted. Other experimenters have maintained that the presence of specific chemical elements in excess has definite morphological consequences.

As regards complex substances and their rôle in morphogenesis, but little is actually known. Recent results indicate that the egg contains substances of complex chemical composition which are essential for the development of specific parts or tissues. Thus certain experiments consist in the removal of definite parts of the egg containing specific materials; and in the subsequent development specific parts of the embryo are wanting. In other experiments, by Conklin, the transference of definite substances from their normal location by means of centrifugal force is followed by the development of corresponding

[2] Stimuli in the sense in which we use the word involve merely the impinging of energies on the stimulated system; if substantive additions are involved we have more than a mere stimulus, to the extent that substances are added to the system.

specific structures in the abnormal location. These experiments strongly suggest, even if they do not rigorously prove, that such substances are essential ingredients in definite developmental processes.

I am indebted to Dr. Riddle for the following illustration: The various colors of mammals, such as black, brown, red, yellow, are due to chemical substances known as melanins. The chemistry of these substances starts out from a simple colorless base or chromogen, from which the series of colors, yellow, red, brown, black, is derived as successive stages of oxidation. The chromogen base is found in all mammals; the color then would appear to be due to the varying powers of the cells of different individuals to oxidize the given base. Tornier has shown in his experiments on the coloration of Amphibia that the particular color developed is a function of nutrition, varying in the order of oxidation value (as was later ascertained) according to the degree of nutrition. The development or inheritance of color, therefore, can certainly not be due to the presence of black or brown or red or yellow determinants in the germ, assumed for theoretical purposes by some students of heredity, but to a specific power of oxidation of the protoplasm. This faculty in its turn is no doubt capable of resolution into other physiological terms.

We are only at the beginning of the study of correlations of embryonic metabolism. The rôle that the internal secretions of the embryo may play in the processes of development is practically unknown; but we may expect to find here biological reactions of fundamental significance, especially when we consider such phenomena of the adult as the influence of pregnancy on the organism, the possibility of inducing lactation, with all that this implies, by injection of fœtal tissues; the relations between the sex organs and secondary sexual characteristics and indeed the entire habitus of the organism; the influence of a small gland like the thyroid, or the pituitary body, etc. Biochemical reaction runs through every phase of development and is unquestionably the decisive factor in the appearance of many characters of the organism.

Self-differentiation.—The conception of self-differentiation in morphogenesis is a vague and unsatisfactory one. In a sense it is a contradiction in biological terms, for assuredly environment enters into every biological process. On the one hand, the term covers the fact of the specificity of primordia, which means only a certain stability of metabolism and reaction capacity; on the other hand, it may have specific meaning in one large class of developmental phenomena, viz., polarization and localization. If, for instance, the term self-differentiation might be applied to the appearance of definite axes, angles, points and faces of a crystal, it would with equal propriety be applicable to the appearance of polarity, bilaterality, etc., the axes of embryonic development. But if the term should come to hold simply this restricted meaning, then all reason for its maintenance would be gone.

It is not at all certain that it will be possible to reduce all the problems of the physiology of development to such categories as we have mentioned. The subject is full of unsolved problems, but so far as I can see no one has shown any real reason for assuming ultra-physical agencies in any of the events, and there is the same pragmatic reason for refusing to assent to such suggestions, which are made all too frequently, that there is in other fields of science. If we will be consistent, we are driven to the conclusion that the apparent simplicity of the germ is real, that the germ contains no gemmules, or determinants or other representative particles; that development is truly epigenetic, a natural series of events that succeed one another according to physico-chemical and physiological laws; the explanation of the sequence consists simply in the discovery of each of its steps.

Applications

The problems of heredity and variation are included in a true physiological conception of the individual development; but some biological conceptions that have more or less status and reputation are inconsistent with it. Such are the inheritance of acquired characters, atavism, and the theory of unit characters. The first is a familiar problem that I shall not argue anew; the second logically implies the presence of ancestral representative particles in the germ, which is inconsistent with a physiological theory of development. But it is obvious that the facts united under the name of atavism or reversion take their place naturally in a physiological theory of development, as arrests of development, or modification of environment, or in other ways.

The theory of unit characters deserves more attention for it is essentially a modern theory, and counts numerous adherents. This conception has been most sharply formulated by De Vries in his Mutationstheorie. He says:

> The properties of the organism are constructed of units which are sharply distinguished from one another. These units may be united in groups, and in related species the same units and groups occur. Intermediates between the units, such as the external forms of plants and animals exhibit so abundantly, are not found any more than between the molecules of chemistry.

Bateson's allelomorphs constitute a similar conception. Such hypothetical elements of organization must be conceived as distinct from the germ on. They can be shuffled about from one generation to another, and can, therefore, be introduced, removed or replaced in the germ cells.

It must be admitted that these conceptions fit certain facts of inheritance in many hybrids fairly well, but the progress of discovery has made necessary the installation of subsidiary hypotheses, so that the most recent conceptions of unit characters are becoming extremely

complex, and it would seem as though the system would soon fall of its own weight. The entire value of the hypothesis consists in the formal approximate expression of certain facts; when it is found that the hypothesis begins to fail even for the classes of facts for which it was originally intended, and that most of the known facts of development can not possibly be expressed in its terms, the entire conception is put on trial.

The weakness in the theory of unit characters is in the use and conception of the term "character.' The term has been prescribed to us by the systematic zoologists and botanists engaged in describing the differences between species; so that "character" really means any definable feature of an anatomical kind that differentiates species; by extension it also means any other differentiating features that can be defined. In the study of evolution and heredity, it is usually only anatomical characters that are in question. Now the study of the physiology of development teaches us that whatever else "characters" may be, they are not units; *they simply represent the sum of all physiological processes coming to expression in definable areas or ways,* and they may thus represent a particular stage of a chemical process, or a mode of reaction of some part. "*Character*" is essentially a static morphological term; in the study of heredity and development we are dealing with biological *processes.* To adapt a phrase of Huxley's: "characters" are like shells cast up on the beach by the ebb and flow of the vital tides; they have a more or less adventitious quality. To give them representation in the germ is equivalent to a denial of uniformity in biological phenomena.

Just as the exact position of each shell on a beach might be fully explained if we knew its full history, so each character has a certain kind of inner necessity as the result of a sequence of developmental processes. And just as in the history of the position of the shell on the beach we should certainly ascribe great importance to the tides and winds, so in the quality of each individual character we should find corresponding vital tides and winds, as regular and lawful as those of the ocean. We do not yet know the secrets of the vital tides; we maintain only that they are the moving forces in development and heredity, just as in physiology and pathology; and every fundamental contribution to the physiology of protoplasm is at the same time, and to the same extent, a contribution to heredity and the physiology of development.

But if these principles are accepted, how are we to explain the facts on which the theory of unit characters depends? The main difficulty lies not in the facts of mutation, for the physiology of this phenomenon already begins to appear from the experiments of Tower and MacDougal, who show that mutations may result from action of

environment directly on the germ cells. The most fundamental phenomena in the unit character theory are unquestionably the segregations of characters that appear in the offspring of hybrids in so-called Mendelian inheritance. In the most typical cases, grandparental characters reappear in definite proportions of the progeny of the hybrid generation. The interpretation, according to the theory of unit characters, is in the hypothesis of purity of the germ cells of the hybrid generation with respect to the segregated characters; which means that the germ cells of the hybrid generation are pure with reference to the contrasting characters united in the soma; in other words, that corresponding contrasted characters can not both remain in the same germ cell, but are segregated in different ones and may thus appear pure in the descendants of a hybrid generation.[8]

We may well doubt that absolute purity of grandparental characters in the offspring of the hybrid generation occurs, and the results unquestionably vary with the environment; but I believe that we have to admit the general principle of segregation. However, the theory of segregation of unit characters in the germ cells is in no way necessary to explain the results; it is in fact inconsistent with the highly variable result; if unit characters were segregated in the germ, we should expect very definite constant results.

If we take our stand on the epigenetic basis and regard the germ cells as no more complex than *direct* investigation would lead us to suppose, then we have to admit that segregation in the germ cells can involve only constituents of the germ cells themselves. But any variation thus induced in the germ cells would be a factor in each process of the development, and would hence tend to influence every character that appears. Such a hypothesis involves the conception that germ cells contain elements capable of segregation; and this is so. Even if the principle of segregation of characters in inheritance had never been discovered, the principle of segregation of germ-cell elements would still hold, for the two discoveries were made absolutely independently.

I refer to the work of Guyer and Montgomery on the chromosomes, which has been followed by a long series of very exact studies. These studies certainly suggest segregation of parental chromosomes in varying proportions in different germ cells. Indeed, I know of no other interpretation of chromosome behavior that is consistent with the facts. Whatever value we may attribute to the chromosomes in cellular physiology, the variable relations established by their differential segregations, even if only quantitative differences are concerned, must involve endless secondary effects in the long series of cell generations that make

[8] Recent modifications of the theory of purity of the germ-cells do not essentially modify the argument.

up the individual life history.[4] It is not impossible that other segregations than those of the chromosomes form part of the germ-cell behavior, but of this we know nothing as yet. In any event, the principle of segregation of actual visible elements of the germ cells has a firm anatomical basis.

It must not be forgotten that the germ is the entire organism and that it passes through development as the same individual; *continuity of individuality is preserved throughout development.* Therefore, if we discard determinant hypotheses and take our stand on a strictly physiological theory of development, it follows of necessity that the transmitted factors of heredity included in the organization of the germ cells must be factors in the development of the entire organism. The so-called Mendelian factors must therefore be of this character, as I have argued elsewhere. That is to say, the segregated factors must be general constitutional conditions effective as factors in the development of every part of the organism. It can readily be seen that specific intensity of metabolism, or of reactivity, and variation in constitutional size of cells may be such conditions. Others no doubt exist, of which sex may be one. The essential thing to recognize is that the heritable and segregable factors, being conditions of the germ cells at the start, can never be anything less than factors of the entire organism at all stages.

Our conclusion is that the theory of individual development must more and more come to be regarded as a branch of physiology proper. The theory of representative particles must be relegated to the class of formal hypotheses whose usefulness is largely outlived. While it may still play a part in speculations on heredity, I believe that it will come to be generally recognized by those who use it as a mere matter of convenience of terminology, and not as an explanation of the phenomena described in its terms, in the sense of being a verifiable part of the sequence of processes in development.

[4] This general argument would stand even if the chromosomes be regarded merely as indices of organization. They at least give us a clue as to what "the organism" is doing at the time in question. This is indeed all we can say of any characters at any period if we consider the matter in a strictly logical sense.

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STUDIES OF FERTILIZATION IN NEREIS

I. THE CORTICAL CHANGES IN THE EGG: II. PARTIAL FERTILIZATION

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TEN FIGURES

ONE PLATE

I. THE CORTICAL CHANGES IN THE EGG

In many animals one of the immediate effects of fertilization is to cause the egg to throw off a membrane, which is therefore known as the fertilization membrane. This is the case for instance in the eggs of echinids and nematodes. In other cases, where a definite vitelline membrane exists prior to fertilization, cortical changes occur in the egg immediately after insemination, leading to the formation of a space, the so-called perivitelline space, between the protoplasm of the egg and the vitelline membrane. This is the case for instance in the eggs of at least many annelids, molluscs and vertebrates, and it is unquestionably a more common phenomenon than the formation of a fertilization membrane. There can be little doubt that these apparently different phenomena are simply varying expressions of a change in the cortex of the egg, which is of the same nature in all cases. Loeb's studies ('09) have thrown much light on the nature of these cortical changes. In the case of the egg of Nereis they are relatively obvious in their character and readily followed.

The ovocyte of Nereis is somewhat flattened in a polar direction, measuring about 87.5 x 100μ; it is girdled by a double equatorial zone of large oil drops. The large germinal vesicle is central and somewhat elongated in a polar direction.

In his description of the unfertilized egg, Wilson ('92) distinguished two membranes: a delicate outer vitelline membrane,

and a subjacent membrane or layer, about 6-7μ in thickness, which he called the zona radiata. As will appear from the sequel however, the latter is not a membrane in the usual meaning of the word, but a cortical, coarsely alveolar layer of the egg. It is transparent and somewhat granular, and the granules tend to be arranged in radiating lines. There is no perivitelline space in the unfertilized egg.

In sections of unfertilized ovocytes fixed in Flemming's fluid, the zona radiata is seen to be a coarsely alveolar layer with homogenous alveolar contents (fig. 1). The walls of the alveoli are continuous internally with the protoplasm of the egg, and unite externally to form a protoplasmic layer applied to the vitelline membrane. The alveoli are closed externally (figs. 1 and 2). The zona radiata is in fact a coarse emulsion or foam-structure.

Unfertilized eggs of Nereis are entirely devoid of jelly and they lie in immediate contact in the sea-water. If India ink be ground up in the water, the particles come in contact with the vitelline membrane. Each *fertilized* egg, on the other hand, is surrounded by a thick layer of colorless transparent jelly; If many eggs are contained in the dish, fusion of the contiguous gelatinous membranes binds the eggs into a mass; the cortical layer (zona radiata) is absent in fertilized eggs, and there is a narrow perivitelline space between the vitelline membrane and the surface of the egg (fig. 3).

The jelly is formed by the extrusion, or diffusion, of the alveolar contents of the cortical layer through the vitelline membrane; the egg of Nereis, in fact, secretes its own jelly, as may be readily demonstrated in life by inseminating under the microscope with excess of sperm. If the sperm be added to closely placed eggs and a cover glass applied so as to force the eggs into a single layer, and the preparation examined with no loss of time, the spermatozoa will be seen in large numbers in contact with the vitelline membrane. In one or two minutes the spermatozoa are moved away from the surface of the membrane by some invisible repelling substance, and, if the eggs be numerous, the spermatozoa unite in three to five minutes to form lines that bound hexa-

gonal areas with the eggs in the centers of the hexagons (fig. *A*). The substance that sweeps the spermatozoa away from the surface of the eggs is the jelly. Synchronously with its formation, the alveoli of the cortical layer are emptied and the alveolar walls now appear as delicate lines crossing a wide perivitelline space[1] (fig. *B*).

However, not all of the spermatozoa are thus carried out by the secreted jelly, but in the case of each egg a single spermatozoon remains attached to the vitelline membrane. This is very prettily demonstrated if the eggs are under some pressure, so that

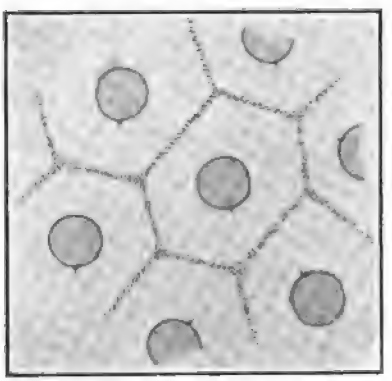

Fig. A. Diagram of fertilization with excess of sperm. The outflow of jelly from the eggs has carried the supernumerary spermatozoa away from the surface of the eggs (see text). In the case of each egg the single effective spermatozoon remains attached. From a sketch of the living object.

the spermatozoa are prevented from reaching the eggs above or below. In this case one can discover the single spermatozoon attached to the vitelline membrane in practically every egg (fig. *A*).

All stages of the disappearance of the cortical layer may be readily and rapidly observed. The alveolar walls, however,

[1]Wilson ('92) states that "from twenty to thirty minutes after fertilization the striae of the zona suddenly become indistinct and in the course of two or three minutes the zona itself entirely disappears, leaving only the outer membrane." But inasmuch as he was under the impression that the unfertilized eggs possess a transparent, thick, gelatinous envelope like the fertilized ones, he failed to observe the interesting phenomenon of formation of the jelly described here.

remain as delicate strands of protoplasm uniting the vitelline membrane to the surface of the egg. The jelly, therefore, represents the alveolar contents only of the cortical layer of the unfertilized egg, and the perivitelline space is nothing but the contracted alveoli of the cortical layer filled with fluid. The perivitelline space must, therefore, be regarded as *intraovular* with a delicate external cytoplasmic wall lining the vitelline membrane; this we may distinguish as the *plasma membrane;* it is comparable in some respects to the fertilization membrane of sea-urchins.

Unfertilized eggs allowed to stand in the sea-water form no jelly and retain the cortical layer; the germinal vesicle remains intact; but, if they be strongly centrifuged, the jelly forms, the perivitelline space arises, the germinal vesicle breaks down and both polar bodies are formed; but parthenogenetic development, usually at least, does not take place. Similarly, the addition of a fairly strong solution of potassium chloride to the sea-water causes formation of the jelly and the perivitelline space while the eggs are still in the solution; maturation takes place after transfer to sea-water but cleavage does not occur (in my experiments; cf. Fischer), though some differentiation may take place without cleavage. It would appear, then, that conditions that so alter the permeability of the plasma and the vitelline membranes as to permit the outflow of the alveolar contents of the cortical layer initiate development, but that the normal continuation of development is dependent on other factors.

In the normal fertilization of Nereis the stimulus of the spermatozoon causes the formation of the jelly and the perivitelline space long before it has penetrated the membrane; in fact penetration does not take place until 40 to 50 minutes after insemination. However, mere *contact* of the spermatozoon with the membrane is apparently not sufficient; but actual *attachment* of at least a single spermatozoon is required; this is shown by the fact that the effective spermatozoon is not carried away from the membrane with the unsuccessful ones by the outflow of jelly. Yet the effect of the localized stimulus of the attached spermatozoon is practically instantaneously effective over the entire

extent of the membrane; it is more like an electrical discharge or some other physical disturbance than a chemical effect.

J. Loeb ('09) has formed the hypothesis that the cortical layer of the egg, especially of sea-urchins, is an emulsion which is rendered stable by a third substance consisting of lipoids, especially cholesterin. The emulsion becomes unstable on solution of the lipoids; this enables the albuminous drops, which he conceives to form one phase of the emulsion, to take up water; hence the layer liquefies and the perivitelline space arises; the fertilization membrane is thus formed. Hence, according to Loeb, the action of lipoid-dissolving substances is to cause the formation of the fertilization membrane. Without committing ourselves to these specific views of the nature of the cortical emulsion, which Loeb himself does not hold very strongly, we may admit that Loeb's hypothesis, that the formation of the fertilization membrane is due to the breaking down of a cortical emulsion, fits the case of Nereis very well. If we go further, however, we must note an important lack of agreement with Loeb's hypothesis. As Loeb himself points out, the theory implies that the membrane of the egg is permeable for sea-water and crystalloid substances, and on the other hand impermeable for colloids; in Nereis the contents of the cortical alveoli are unquestionably colloid, as Loeb's hypothesis requires, but it is perfectly certain that they diffuse through the membrane to form the external jelly; at the same time, unquestionably, sea-water enters to take the space previously occupied by the colloid. The membrane is therefore permeable for both crystalloids and colloids at this time. I have not, however, investigated farther the properties of the egg membranes and must leave this problem to those who are better qualified as physiologists to make such a study.

It would appear that the presence of this colloid substance in the cortex is an inhibition to the maturation of the egg, because as soon as it is removed, maturation processes are set in motion and both polar bodies are formed. In what manner it inhibits is of course problematical. In the egg of Ascaris megalocephala there is a similar excretion of a cortical colloid which forms, in this case, the thick resistant perivitelline membrane. The ap-

pearance of the fertilization membrane of echinids might be similarly due to excretion of a cortical colloid which is removed by diffusion and hence is not detected. It is a problem worthy of careful investigation whether the loss of cortical colloids is not the first step in fertilization generally.

II. PARTIAL FERTILIZATION

Two functions of the spermatozoon in fertilization may be sharply distinguished. The first is the initiation of the development and the second is the transfer of paternal qualities to the fertilized ovum (heredity from male parent). The first function alone is under consideration in these experiments.

We have seen that in Nereis the immediate effect of attachment of the spermatozoon is essentially the same as a mechanical shock (centrifuging), or a chemical stimulus (KCl); that is, it causes the breaking down of the cortical emulsion and consequent formation of the gelatinous envelope of the egg. But apparently the resemblance extends no farther, for in the case of mechanical or chemical stimulation the impulse to development is lost or greatly weakened after maturation has occurred; and the eggs do not segment. On the other hand the normally fertilized egg does not stop after maturation, but proceeds with its development in a normal fashion. Now the cause of this difference might be either: (a) because the stimulus of the spermatozoon is qualitatively different from, or stronger than, mechanical or chemical stimulation, or (b) because the fertilizing action of the spermatozoon is not completed with the cortical changes but continues after its entrance into the egg. If the first alternative were correct, then the elimination of the spermatozoon after membrane formation should not prevent the normal cleavage and development of ova which had once been stimulated by it; but if the second alternative were correct and the sperm nucleus were prevented from entering the egg after it had induced membrane-formation, then such ova should proceed no further in their development than those mechanically or chemically stimulated.

I have been able to perform this experiment on the eggs of Nereis and have found that eggs in which the spermatozoon is removed after the cortical changes have occurred proceed but little farther in their development than eggs mechanically or chemically stimulated, and they do not undergo segmentation. Fertilization is therefore still incomplete after the formation of the fertilization membrane.

It will be seen that if the results above indicated be demonstrated, the process of fertilization is obviously something more than a beginning of cytolysis or a mere alteration of permeability of the peripheral cell membrane. It would appear to be a progressive change, starting at the periphery and gradually involving the more central portions of the cell. We would, at least, have to distinguish two stages in the fertilizing action of the spermatozoon, one before and the other after penetration.

I shall consider first the evidence for the statement that in the egg of Nereis elimination of the spermatozoon after membrane-formation leaves the process of fertilization incomplete. In the second place I shall note the respects in which the completely fertilized egg differs from the partially fertilized egg, and finally, shall consider the bearing of the facts on the theory of fertilization. Inasmuch as it will be necessary to make frequent comparisons with the normal fertilization, a brief account of the salient features of this process will be given first.

A. Salient features of the normal fertilization

The egg of Nereis is difficult to fix in a thoroughly satisfactory fashion; owing, no doubt, to the presence of the large oil-drops and yolk-granules, uneven fixation with shrinkage is hard to avoid. The eggs appear likewise difficult of penetration, owing probably to the rather viscid jelly from which they cannot be separated; this also makes any considerable number of eggs very bulky and the killing fluid is apt to be much diluted if used in ordinary amounts. After considerable experimenting with picric acid, corrosive sublimate and osmic acid fixing fluids, I finally found one which gives practically perfect results in all

stages of maturation and fertilization. This is Meves' modification of Flemming's fluid made as follows: chromic acid, 0.5 per cent, 15 cc.; osmic acid, 2 per cent, 3.5 cc.; glacial acetic acid 3 drops. The eggs were left in the fluid from thirty to forty-five minutes. Fixation in this fluid causes no shrinkage, the oil is so changed that it is not dissolved out by subsequent imbedding in paraffine; the sections stain beautifully in iron haematoxylin, and certain substances are clearly differentiated which can be detected only with the greatest difficulty after fixation in any other fluid tried.

a. The penetration of the spermatozoon. It was noted in the first part of this paper that a single spermatozoon becomes attached to the egg-membrane immediately after insemination, and that the breaking down of the cortical layer, secretion of the jelly and formation of the perivitelline space follow immediately, though the actual penetration of the spermatozoon is delayed forty or fifty minutes.

The act of penetration involves no motile activity on the part of the spermatozoon; after the latter has become attached to the vitelline membrane all movement of the spermatozoon ceases and it remains absolutely immotile throughout the forty or fifty minutes that elapse before it is taken into the egg. The events of this period as seen in the living egg are as follows:

1. The jelly is formed by outflow of the alveolar contents of the cortical layer as already noted; although a large amount of jelly is formed in two or three minutes, yet the process lasts ten or fifteen minutes before the deeper alveoli are emptied. There is then a very wide perivitelline space crossed by the alveolar walls which are attached to the plasma membrane, presenting a very striking appearance (fig. B).

2. The protoplasm of the egg immediately beneath the attached spermatozoon then forms a rounded elevation, the entrance cone, which gradually moves across the perivitelline space and comes in contact, and fuses, with the membrane (fig. B, a). This condition is usually fully attained about fifteen to seventeen minutes after insemination.

3. The entrance cone then gradually retracts, drawing the membrane down to form a depression in which the spermatozoon is included. At this stage one may easily imagine that the sper-

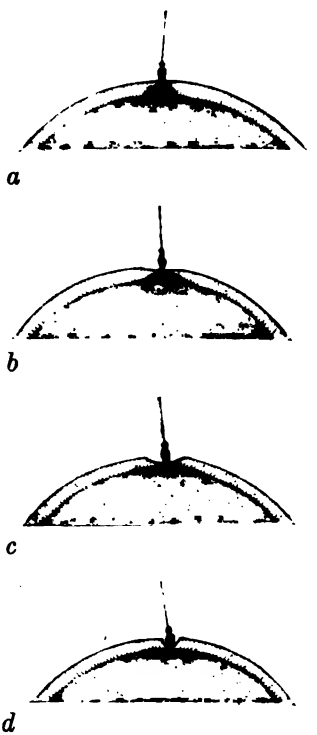

Fig. B. History of the fertilization-cone as seen in the living egg. Four camera drawings of the same egg:—

a Seventeen minutes after insemination,
b Nineteen minutes after insemination,
c Twenty-two minutes after insemination,
d Twenty-four minutes after insemination,

The fertilization-cone is shown at the height of its development in *a*, its gradual recession and the simultaneous formation of a depression in the membrane is shown in *b*, *c* and *d*.

matozoon has been taken into the egg, as it is apt to be concealed in the depression of the membrane; but this is not the case. The stage of best development of the depression, corresponding to

the complete retraction of the entrance cone, is about twenty-two to twenty-five minutes after fertilization (fig. B, b, c, d).

4. The perivitelline space then narrows around the entire egg, and the depression of the membrane in which the spermatozoon is seated disappears; in consequence, the spermatozoon again becomes prominent externally.

5. It remains prominent for ten or fifteen minutes (about forty to fifty minutes after insemination), and then disappears rather abruptly within the egg as though some resistance had given away. Its penetration coincides with the late anaphase of the first maturation division; in a few cases it may be a little earlier or a little later.

The egg is changing form at this time and in consequnce the perivitelline space is often widened locally, especially in the animal hemisphere; if this happen in the region of penetration, which may be any part of the egg, strong cytoplasmic strands are drawn out between the membrane of the egg and the point of penetration, showing that the egg membrane and the cytoplasm are actually fused here.

To repeat and extend the observations on the living egg several series of eggs were preserved every five minutes from the time of insemination in Meves' fluid. The study of the sections confirmed and extended the above observation on the living egg as follows:

Ten minutes after insemination the entrance cone is quite well formed and the spermatozoon is clearly seen outside, separated from the entrance cone only by the thickness of the vitelline membrane with which it is in contact.

Fifteen minutes after insemination essentially the same condition persists. The entrance cone is homogeneous, shading off into the surrounding yolk-filled protoplasm. It stains very dark in iron haematoxylin. The head of the spermatozoon appears exactly as before.

Twenty minutes after insemination the entrance cone has flattened out, but the spermatozoon is still external to the membrane. The substance of the entrance cone is, however, as readily recognized as when it projected above the surface of the egg.

About thirty-seven minutes after insemination (metaphase of first maturation division) the sperm is still readily found on the exterior of the vitelline membrane external to the substance of the entrance cone which is now lens shaped. The substance of the entrance cone is homogeneous and it stains less than before; it is sharply marked off from the unaltered egg cytoplasm by a layer of small basophile granules. In the center of its external face is a sharply differentiated granule which stains intensely black in iron haematoxylin, and which is connected to the sperm head by a fine thread passing through the vitelline membrane; penetration has already begun.

Forty-three minutes after insemination (late metaphase of the first maturation division) the entrance cone sinks into the egg-cytoplasm, and the head of the spermatozoon begins to be drawn within the egg in the form of a thick thread, less than one-third the diameter of the sperm head, however. The sperm nucleus is being drawn through the small perforation in the vitelline membrane.

Forty-eight minutes after insemination (stages of anaphase of the first maturation division), nearly all of the sperm head is drawn into the egg in the form of a thick thread several times longer than the original sperm head. Before the head is entirely within the egg its inner end begins to swell and becomes vesicular. The entire entrance cone penetrates the egg-protoplasm always retaining its original connection with the apex of the spermatozoon, so that the original orientation of the sperm is preserved and may be readily recognized after penetration.

Fifty-four minutes after insemination (telophase of the first maturation division), the entire head of the spermatozoon is within the egg. The tail and middle piece usually remain without.

As I intend to publish a separate account of the interesting details of penetration of the spermatozoon, and as the later stages do not concern the present problem, I shall simply say, therefore, that as the united sperm-head and entrance cone penetrate farther into the egg cytoplasm, they rotate in such a way that the entrance cone which was originally in advance of the sperm nucleus comes to lie behind it. During the rotation the sperm

aster arises from the pole of the sperm nucleus opposite the entrance cone, thus in the position of the original middle piece.

Morgan has recently ('10), with entire justice as it appears to me, taken a stand against the current view that penetration of the sperm is due to mechanical boring into the egg. He believes that the presence of the sperm calls forth a reaction on the part of the egg that leads to the absorption of the former. There can be no question that the latter conception fits the case of Nereis much better than the former. In the first place the spermatozoon is absolutely motionless after its attachment to the membrane; in the next place the formation of the entrance cone shows a very decided reaction on the part of the egg to the presence of the spermatozoon; in the third place the retraction of the spermatozoon into a depression of the membrane is due to the retraction of the entrance cone; and finally, as I shall show in a subsequent cytological study, the inclusion of the spermatozoon within the egg appears to be due to activity of the substance of the entrance cone, and not to active penetration by the spermatozoon. The spermatozoon does not penetrate the egg, it is drawn in or engulfed.

b. The later history of the sperm nucleus. The sperm amphiaster is visible in the preparations all through the period of the second maturation division (fig 4). After the formation of the second polar body the sperm-nucleus begins to enlarge and the amphiaster gradually wanes, but it may be recognized up to the time of contact of the germ nuclei. The centrosomes of the first cleavage spindle then begin to appear. Whether or not they are continuous with those of the sperm amphiaster is a question which I shall take up in the next study of this series. The cleavage asters rapidly become very large and conspicuous (figs. 5 and 6). During the growth of the germ nuclei a considerable number of large granules staining strongly in iron haematoxylin appear in their immediate vicinity.

The main point of these observations on the normal fertilization, both in the living eggs and also in section, is to demonstrate for elucidation of the experiments following: (1) That membrane formation precedes penetration of the spermatozoon by a long time. (2) That the spermatozoon does not penetrate

the vitelline membrane and enter the egg until at least forty to fifty minutes after insemination, although its attachment to the membrane takes place immediately. (3) That the presence of the sperm-nucleus is readily demonstrable in all stages after penetration.

B. Removal of the spermatozoon after membrane formation

In the summer of 1909 I was studying the effects of centrifuging on the egg of Nereis with the aim of getting more data on the problem of polarity and the theory of formative stuffs. It soon became apparent that the effects of centrifuging varied with the stage of development, and so several series of experiments were made in which the eggs were centrifuged at regular intervals from before fertilization up to the time of the first cleavage.

The effects of centrifuging may be divided into three categories: (1) A certain proportion of centrifuged eggs develop approximately normally, the percentage varying greatly with the stage of centrifuging. (2) A certain proportion of eggs, varying at different stages, segment more or less abnormally, sometimes extremely so (*e.g.* meroblastic), and produce embryos with more or less pronounced abnormalities. (3) At certain stages of centrifuging a variable proportion of eggs fails to carry out even the first cleavage. The investigation of the causes of such failure to segment revealed the fact that it was owing to the removal of the spermatozoon after membrane-formation. It is the evidence for this statement that is now under consideration.

The results with reference to failure to segment were, in general, as follows:

1. If unfertilized eggs were centrifuged and then fertilized, all segmented, and a large percentage tended to develop quite normally.

2. A disturbing factor comes in shortly after insemination, owing to the fact that when the jelly is first secreted by the eggs it is so viscid that the eggs stick together in the bottom of the centrifuge in a mass which cannot be separated into its constitu-

ent eggs. The extreme viscidity of the jelly gradually disappears, and after about twenty minutes from insemination, the eggs no longer mat together. It is therefore difficult to investigate the effects of centrifuging on the developmental capacity of the eggs during the first ten or fifteen minutes after insemination. However, when the viscid stage begins to pass away and eggs can be separated from the mass for examination, the majority are found to undergo segmentation, as many as 98 per

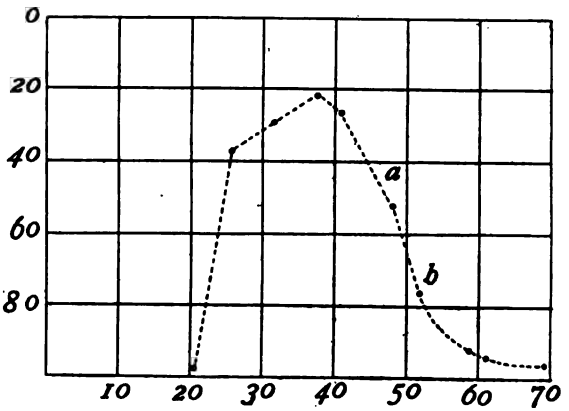

Fig. C. The effects of centrifuging on the power to segment in Nereis. The abscissae represent minutes from the time of i semination, the ordinates the percentage of eggs dividing. At position *a* penetration of the spermatozoon is just completed in most of the eggs. At position *b* the first polar body is extruded. Data from experiment 2, 1910.

cent in one case (experiment 2, 1910) twenty-one minutes after insemination.

3. For about the next thirty minutes (twenty-one to fifty-three minutes after insemination) centrifuging tends to inhibit the cleavage of a certain proportion of the eggs which gradually increases up to about thirty-seven minutes after insemination and then decreases again. For instance, in experiment 2 of 1910, of the eggs centrifuged twenty-one minutes after insemination 98 per cent segmented; twenty-six minutes after insemination 36 per cent segmented; thirty-two minutes, 33 per cent; thirty-seven minutes, 21 per cent segmented; forty-three minutes, 26 per cent segmented; forty-eight minutes, 52 per cent segmented; fifty-three minutes, 75 per cent segmented; fifty-

eight minutes, 90 per cent segmented; sixty-three minutes, 90+ per cent segmented; sixty-nine minutes, 95+ per cent segmented; control eggs, 99 per cent segmented. (See Fig. C.)

4. From this time on nearly all of the eggs segment after centrifuging until, during the anaphase and telophase of the first cleavage spindle, centrifuging again tends to inhibit cleavage.

The following table (Experiment 29) gives a fairly typical series of results. There were twelve such experimental series in all, more or less complete, giving concordant results except that in some at the period corresponding to 29D, 90 to 98 per cent were so affected that they failed to segment. On either side of this critical period there is a decreasing susceptibility to such injury by centrifuging.

EXPERIMENT 29

September 8, 1909

DESIGNATION	TIME OF CENTRIFUGING AFTER INSEMINATION	PERCENTAGE SEGMENTED	REMARKS
29	Control (not centrifuged)	100 per cent	
29A	Before insemination	100 per cent practically	
29B	20 minutes	Majority	Eggs matted loosely
29C	30 minutes	Majority	
29D	41 minutes	20 to 30 per cent	
29E	51 minutes	70 to 80 per cent	
29F	66 minutes	90 to 95 per cent	
29G	79 minutes	100 per cent	
29H	95 minutes	100 per cent	
29I	114 minutes	100 per cent	
29J	121 minutes	Some unsegmented	
29K	127 minutes	Less than majority and these irregular	Centrifuged during process of 1st cleavage
29L	149 minutes	Most segmented further	Centrifuged in 2-cell stage

Two major processes are going on in the egg at this time, viz.: maturation and fertilization. The injury is not primarily to the process of maturation, for the eggs that do not segment form the polar bodies; nor is it probable that there is a general

systemic injury to the egg protoplasm at this time not received at other times, when the fact that maturation continues and polarity is preserved in these eggs, is considered. It would, therefore, appear probable that the injury is to the process of fertilization itself, and this conjecture is completely confirmed by cytological study. The most conclusive experiment in this respect is no. 27, the details of which are as follows:

The eggs were fertilized at 9:28 A.M., September 4, 1909. Some of these were kept for control and all segmented normally. The remainder were centrifuged about 60 x 120 revolutions at a radius of 6 cm. in one minute, at the following times: 27A at 9:58 A.M.; 27B at 10:03; 27C at 10:08; 27D at 10:12; 27E at 10:16. About 20 per cent of 27A segmented, 5 to 10 per cent of 27B, 20 per cent of 27C, 50 to 60 per cent of 27D, and 75 to 90 per cent of 27E. Samples of the control and of each of 27A, 27B, 27C, 27D and 27E were preserved at 10:31 and 10:43 A.M.[2]

EXPERIMENT 27

Eggs fertilized at 9:28 A.M., Sept. 4, 1909

DESIGNATION	CENTRIFUGED	TIME AFTER INSEMINATION	SAMPLES PRESERVED	LIVING EGGS
27 Control..	Not centrifuged		(1) 10:31½ A.M. (2) 10:45 ½A.M.	All segmented
27A	60 x 120 rev. in 1 min. 9:58 A.M.	30 min.	(1) 10:30 A.M. (2) 10:43½ A.M.	About 20 per cent segmented
27B....	60 x 120 rev. in 1 min. 10:03 A.M.	35 min.	(1) 10:30½ A.M. (2) 10:43½ A.M.	About 5-10 per cent segmented
27C....	60 x 120 rev. in 1 min. 10:08 A.M.	40 min.	(1) 10:30½ A.M. (2) 10:44 A.M.	About 20 per cent segmented
27D....	60 x 120 rev. in 1 min. 10:12 A.M.	44 min.	(1) 10:31 A.M. (2) 10:44½ A.M.	About 50-60 per cent segmented
27E....	60 x 120 rev. in 1 min. 10:16 A.M.	48 min.	(1) 10:31 A.M. (2) 10:45 A.M.	About 75-90 per cent segmented

[2]Since the above was written this experiment has been repeated (Exp. 2, '10), with the added precaution of preserving a sample of the normal eggs corresponding to each stage of centrifuging, in order to make certain of the stages of fertilization in each case. The results completely confirm those of experiment 27, and the sperm was found to be external in the most critical stage (37 minutes after insemination in this experiment; see page 371).

Cytological study of the twelve lots of preserved eggs showed stages ranging from the metaphase of the second maturation spindle to the prophase of the first cleavage, the earlier stages of course being found in lot 1 in each case.

In the control lots it was easy to demonstrate the sperm nucleus at all stages to the formation of the first cleavage spindle. The sperm nucleus is rendered particularly conspicuous during the second maturation division by the large amphiaster that accompanies it (fig. 4), both lying in the yolk-free protoplasm. After the formation of the second polar body the sperm amphiaster gradually fades, but the sperm nucleus can be recognized by its position and by the remnants of radiations up to the time of union of the two germ nuclei; and in the later stages its presence may be inferred by the degree of development of the cleavage amphiaster and the number of chromosomes. There is, therefore, no time from the beginning of the second maturation division up to the formation of the first cleavage spindle when the presence of the sperm nucleus cannot be readily demonstrated.

In the study of the serial sections of the control eggs I found no egg in which, all sections being present, the sperm nucleus could not be demonstrated. In the serial sections of 27A, the sperm nucleus could be recognized in only about 37 per cent of the eggs; in 27B in only 10 per cent to 20 per cent; in 27C in about 25 per cent; in 27D in about 53 per cent; in 27E in about 76 per cent. The stages of maturation of lots A to E corresponded very closely with the stages of maturation of the control eggs killed at the same time.

It is a relatively simple matter to demonstrate the presence of the sperm nucleus, for a single positive observation suffices; but, to be sure of the absence of a sperm nucleus from any particular egg, it is necessary to examine practically every section of the egg, and the absence of two consecutive sections is sufficient reason for excluding an egg from the count. This may be one reason why the number of eggs in the different lots shown to contain sperm nuclei tends to be somewhat larger than the estimate of the number of eggs that segmented. Another reason

probably is that a sperm nucleus may persist to a certain extent even if injured and unable to produce the full fertilizing effect and cause cleavage.

A third reason for discrepancy in the results is that abnormalities of maturation may be produced by centrifuging which render the determination of the sperm nucleus more difficult than usual. It frequently happens that, as the first maturation spindle is driven from the surface by the centrifugal force, it divides before it reaches the surface again, producing two maturation nuclei within the egg. The two second maturation spindles may then unite to form a tetraster, one pole of which approaches the surface and a single polar body is formed, leaving three nuclei within the eggs (fig. 7). Such eggs were readily recognized by the absence of the first polar globule, and by the presence of the extra nuclei. But it was sometimes difficult to determine in certain stages whether there were only three nuclei, the sperm nucleus being absent, or four, the sperm nucleus being present.

A fourth reason for a certain discrepancy between the estimate of the number of eggs that segmented and the number determined to have sperm nuclei might be that at the time the experiment was made the importance of *exact* determination of the number of eggs that segmented was not realized, and the determination was made only roughly. Putting the results side by side we have:

	PERCENTAGE OF EGGS OBSERVED TO DIVIDE IN LIVING CONDITION	PERCENTAGE DETERMINED BY SERIAL SECTIONS TO POSSESS SPERM NUCLEI
27 Control	All	All
27A	About 20 per cent	About 37 per cent
27B	5 to 10 per cent	About 10-20 per cent
27C	About 20 per cent	About 25 per cent
27D	About 50-60 per cent	About 53 per cent
27E	About 75-90 per cent	About 76 per cent

Considering the various sources of error, the agreement is very close except in 27A. But in this case we do not have to

explain why eggs in which the sperm nucleus was absent segmented but on the contrary, why certain eggs that possessed the sperm nucleus failed to segment, which is a very different thing. There is no evidence that any egg in which the sperm nucleus was absent succeeded in dividing.

The general conclusion that removal of the spermatozoon at the times noted in the experiments involves incomplete fertilization, is sufficiently demonstrated by the results.

Let us call the stage at which the spermatozoon is eliminated in the greatest proportion of eggs, the critical period. The exact number of minutes from the time of mixing eggs and sperm to this stage varies of course through the season, owing to the variations of temperature. Moreover, it is not *exactly* determined in all experiments, for in some the stages of centrifuging fall on either side of it. This being understood, we may note that in eight experiments the critical period occurred at from 25 minutes to 40 minutes after fertilization. This is quite a wide variation, but when the time is represented as a fraction of the entire period between fertilization and the first cleavage, it is found that in all cases the period up to the critical period is between 27 and 33 per cent of the total time up to the first cleavage. It is obviously a corresponding stage in all cases, for the observed differences fall within the chances of error, viz: that the critical period is hit exactly in only very few experiments, and that the time of beginning of the first cleavage must be stated rather arbitrarily on account of the variation in rate of individual eggs.

The critical period occurs shortly before the penetration of the spermatozoon into the egg. We noted in the first part of this paper that the penetration of the spermatozoon is extremely gradual; my observations on this point, both from the study of the living egg and also of sections, show that it requires forty to fifty minutes for the head of the spermatozoon to disappear through the membrane.

As the most critical period comes in the great majority of experiments from thirty-five to forty minutes after insemination, it is obvious that the spermatozoon is in some way prevented

from entering the egg. The explanation is comparatively simple; the spermatozoon is imbedded in the jelly by which the egg is surrounded. When the jelly is first formed it is very viscid, and adheres to the eggs during centrifuging so that they mat together in the centrifuge. However, this stage passes and the result of centrifuging is then to separate the jelly from the eggs. In many cases the jelly carries off the attached spermatozoon with it. After penetration this can of course no longer happen. The curve of variation of the per cent of eggs centrifuged before penetration that fail to segment is due to the following factors: (*a*) At about twenty-five minutes the sperm head is sunk in a deep depression of the membrane and hence is less likely to be torn away by the jelly; (*b*) the change in consistency of the jelly presumably extends from without inwards; hence at first the innermost layer in which the spermatozoon is imbedded tends for a time (also presumably) to remain with the egg; (*c*) the time of penetration varies somewhat in any lot of eggs. These facts together would explain why the percentage of eggs that fail to segment after centrifuging rises to a maximum and sinks again to a minimum in the successive stages of centrifuging.

The fact that centrifuging inhibits cleavage in a small per cent of the eggs from fifty to fifty-five minutes after insemination, leads me to suspect that in some cases the sperm may be destroyed after its penetration into the egg. In experiment 2, 1910, for instance, cleavage was inhibited in 25 per cent of the eggs centrifuged fifty-three minutes after fertilization; in the control eggs killed at the time of centrifuging, penetration of the sperm is completed in the great majority of eggs, though it is found external still in a very few; it is difficult to estimate the per cent of the latter, but the impression is that it is less than 25 per cent. However, it is impossible to confirm this, and I mention the matter here to call attention to the error in my first paper read before the Central Branch of the American Zoological Society (Abstract in Science, vol. 18, p. 36, May, 1910), in which I stated that the destruction of the sperm nucleus followed penetration. A renewed study of the penetration has proved that this is not the case, usually at least.

We are not, of course, free to infer that fertilization is complete as the stimulus to development immediately after penetration of the spermatozoon. The experiments prove directly that in the egg of Nereis the stimulus of the spermatozoon as the impulse to development consists of two distinct parts: (1) an external stimulus that causes membrane formation and which is sufficient of itself to induce the maturation; (2) an internal stimulus dependent on penetration of the spermatozoon. How long after penetration fertilization is still incomplete cannot be decided on the basis of these experiments.

In concluding this section, we may note that the cause of failure to segment following centrifuging during the anaphase of the first cleavage is an entirely different one. The cause in this case is the breaking up of the karyokinetic figure and function, and dispersing the chromosomes. This is readily demonstrated in sections. In the case of eggs centrifuged at the 'critical stage', the sections show that the maturation spindle receives no injury from centrifuging, but appears coherent and normal in the sections. The sections of eggs centrifuged during the anaphases of the first cleavage show the chromosomes dispersed through the cytoplasm and the cleavage spindle no longer coherent, but broken up. In the first case the cause of failure to segment is elimination of the sperm-nucleus, as shown by study of series 27. This cannot be the cause in the second case, and a sufficient explanation of the failure to segment is found in the destruction of the karyokinetic figure.

C. Comparison of completely and partially fertilized eggs in later stages

We have noted so far that definite proportions of eggs centrifuged at definite periods in the process of fertilization fail to develop a sperm-nucleus, and that similar proportions of the same lots of eggs when left to develop fail to undergo segmentation. The facts (1) that all the control eggs of the same lot segment, and (2) that the centrifuged eggs that fail to segment, nevertheless had formed the fertilization membrane and under-

gone maturation, prove that the unsegmented eggs had received at least the first stimulus of fertilization. It was also shown that the critical period for suppressing segmentation by centrifuging occurs at a time shortly before entrance of the spermatozoon, and that it is due to prevention of penetration. The partially fertilized eggs, therefore, resemble the normal ones in the fact that membrane formation and the first stimulus to development are called forth by action of the spermatozoon, and they differ from the normally fertilized eggs in that the internal egg protoplasm has not received the direct stimulus of the spermatozoon. A cytological examination of such eggs could not fail to be of interest and might give some clue to the internal function of the spermatozoon in fertilization.

Both polar bodies form regularly in such eggs as already noted, and the egg-nucleus (female pronucleus) arises and attains the same size as in normally fertilized eggs. The chromosomes of the first cleavage spindle then form in the usual fashion and at the usual time, accompanied by disappearance of the nuclear membrane. But, whereas, in the presence of a sperm nucleus, cytoplasmic asters accompany these processes and a spindle rapidly arises during the prophases of the first cleavage, in the absence of the sperm nucleus there is absolutely no sign of cytasters or evidence of spindle formation. The chromosomes lie naked in the cytoplasm surrounded by a clear area (fig. 7).

Each chromosome then splits longitudinally in the usual fashion, but the halves do not separate. At the time of the telophase of the normal first cleavage there is a tendency to scattering and breaking up of the chromosomes. When the normal eggs have reached the two and four-celled stages, the scattering and breaking up of the chromosomes have progressed much farther in the unsegmented eggs, and in the course of two or three hours there remains no differentiated nucleus or chromosomes, but only numerous chromatic granules scattered throughout the cytoplasm.

The behavior of the partially fertilized eggs may be compared on the one hand with that of normally fertilized eggs and on the other with that of eggs caused to mature by centrifuging. As com-

pared with the former, the chief difference observable by cytological methods is the entire absence of the achromatic part of the karyokinetic figure. The differences in later stages may be conceived as secondary effects of this defect or of the conditions determining such defect. When eggs are caused to mature by centrifuging the process begins as in normally fertilized eggs by the breaking down of the cortical layer and formation of the jelly; the germinal vesicle ruptures and the two maturation divisions follow. After the completion of the maturation the chromosomal vesicles of the egg nucleus usually fail to unite perfectly, and in a little while they separate and scatter in the cytoplasm without formation of chromosomes, so that each egg appears to possess a considerable number of small nuclei. In a few cases the first indications of chromosome formation may be observed in the vesicles shortly after maturation but not later. Subsequently the chromosomal vesicles appear to dissolve in the cytoplasm liberating small chromatic nucleoli.

The partial stimulus of the spermatozoon is thus somewhat more effective than the mechanical shock of centrifuging, though both produced the same initial changes, apparently equally well. This may possibly be due to entrance of a small amount of matter from the spermatozoon; for at the critical period the perforatorium of the sperm has penetrated the membrane and is imbedded in the entrance cone.

D. General discussion

The general conclusion that the function of the spermatozoon in the stimulus to development involves at least two factors has already been clearly stated by Boveri ('07) and Loeb ('09*b*): According to Loeb, one factor is the cytolysis of the "very thin cortical layer of egg"; but while this stimulates development, the latter is often abnormal and therefore usually comes to a halt. A second process is necessary to ensure more normal and lasting development (Loeb '09*b*). Apparently Loeb is not very clear concerning the nature of the second factor, but is inclined to regard it as inhibiting the cytolysis which he conceives to be

begun as the first factor of the developmental stimulus. This conclusion was formed as a result of two kinds of experiments: In the first Loeb found that the best results in artificial parthenogenesis are obtained, in the egg of a Californian sea-urchin, by a double treatment: first using a cytolytic agent and then following it by treatment with hypertonic sea-water, or by inhibiting oxidation for a while. In the second class of experiments Loeb and Elder found that mere membrane formation might be induced in sea-urchin eggs by external contact of starfish spermatozoa, but farther development did not take place except in the relatively few cases in which the spermatozoon entered the egg (Loeb '09*b*, p. 249), or unless the eggs were treated after membrane formation with hypertonic sea-water. Although this experiment is complicated by the hybridizing, yet it demonstrates the same distinction between external and internal functions of the spermatozoon in fertilization that I have shown for Nereis by a different method.

Artificial parthenogenesis may be induced in the sea-urchin egg without membrane formation and this fact appears to me to indicate that the internal function of the spermatozoon is probably at least as fundamental as the external function (membrane formation), though, as Loeb points out, development without membrane formation takes place in a less normal fashion than after membrane formation. But inasmuch as we may have membrane-formation without development, and development without membrane formation, it would seem premature at least to consider membrane formation as the chief function of the spermatozoon in fertilization.

The experiments described in this paper show that in Nereis fertilization by the spermatozoon is incomplete after the formation of the membrane. The question then arises, when is the function of the spermatozoon in fertilization completed? Ziegler's and Wilson's experiments show that it is incomplete even some time after entrance of the spermatozoon. Ziegler's experiments ('98) consisted "in so constricting the egg of the sea-urchin after penetration of the spermatozoon that the one part contains the sperm nucleus, the other part the female sex-nucleus. The

part that contains the sperm nucleus undergoes cleavage and develops farther; in the other part the female sex-nucleus undergoes remarkable transformations, dissolving and reappearing, a process which is repeated several times." In spite, therefore, of the presence of the sperm-nucleus in a constricted part of the same egg, the part containing the egg nucleus was not fully fertilized. It made abortive attempts at division, but the karyokinetic figure was too feeble to carry the process through.

Wilson observed ('03) that if the fertilized eggs of Cerebratulus be cut in two shortly after the penetration of the spermatozoon "only a single fragment develops even though the fragments be refertilized immediately after the operation. In such cases it is almost invariably the nucleated fragments that develop, but in a very few cases I have observed that the enucleated fragment develops, while the nucleated one forms the polar bodies, but proceeds no further." By the nucleated fragment in this case Wilson means the fragment containing the maturation spindle. Farther on he adds "The few cases in which the enucleated fragment of the bisected fertilized egg develops are doubtless those in which the plane of section separates the sperm-nucleus from the egg-nucleus." This is indeed the only possible explanation. In such cases the fragment containing the egg-nucleus is only partially fertilized.

Boveri has also observed that if freshly fertilized sea-urchin eggs be broken into fragments by shaking, certain of the fragments contain the egg nucleus alone. If such fragments are not subsequently entered by a spermatozoon, the nucleus enlarges, dissolves and reappears again; but they do not segment ('96). Later he observed that such pieces may divide at least twice ('02).

These experiments demonstrate that fertilization is still partial even some time after entrance of the spermatozoon into the egg; but they do not show at what stage it is complete. Boveri's very interesting observations on 'partial fertilization' in the sea-urchin egg ('88 and '90) carry the solution of the problem a step farther (cf. also Teichmann '02). In the experiments which furnished the material for his observations both eggs and sperm were weakened, the former by standing for fourteen hours

in sea-water and the latter by treatment with KOH prolonged to a stage in which only a small percentage of the spermatozoa continued to move. Under these conditions in a large number of eggs the sperm aster separated from the sperm nucleus, which was usually left on one side, and proceeded alone to conjugate with the egg-nucleus. Thereupon the cleavage spindle formed with the egg-nucleus alone, and segmentation of the egg ensued. In the four-cell stage usually, but sometimes in the two or eight-cell stage, the sperm nucleus united with one of the segmentation nuclei. Boveri concludes from this and other results that the fertilizing action of the spermatozoon consists in the introduction of a centrosome into the egg.[3] When this has united with the egg nucleus, with or without participation of the sperm nucleus, fertilization would be complete; or with the sperm nucleus alone in merogony it likewise completes fertilization.

Boveri has used the term 'partial fertilization' for the phenomenon just described, although he admits that it is a misnomer. It is unfortunate that such a significance should have come to be attached to the expression, because, as has been shown, my own results and those of Ziegler, Wilson and Boveri himself prove that partial fertilization in the literal sense really occurs. The various stages of partial fertilization as shown by the results in the literature on the subject are:

1. External contact alone by the spermatozoon producing,

a. Formation of the fertilization membrane, (Loeb and Elder for sea-urchins, Lillie for Nereis).

b. Maturation and formation of the chromosomes from the egg nucleus without spindle, (Lillie for Nereis).

c. Maturation and cleavage to stereoblastula, (Bataillon: hybrid union of eggs of Pelodytes punctatus and Bufo calamita with sperm of Triton alpestris).

2. If the spermatozoon be removed shortly after entrance,

a. Maturation alone may result, (Wilson on Cerebratulus).

[3]Herbst ('07 p. 202 and '09 p. 277) interprets Boveri's 'partial fertilization' as a combination of parthenogenesis and fertilization. Such an interpretation does not, however, explain Boveri's account of the behavior of the sperm-centrosomes.

b. An abortive karyokinetic figure may form with the egg nucleus alone, (Ziegler and Boveri on sea-urchins).

c. In some cases at least two cleavages may result, (Boveri on sea-urchins). Boveri's so-called 'partial fertilization, is really full fertilization, so it does not come in this series.

The difference in reaction of the egg-cytoplasm to its own nucleus and to the introduced part of the spermatozoon can be explained on only one of two grounds; either in the general sense of Boveri's theory on purely morphological grounds, or on the ground of a chemical difference, presumably of sexual origin, between the egg on the one hand and the sperm on the other. The latter form of interpretation seems to me preferable because it is a physiological interpretation which takes cognizance of the sexual factor in fertilization.

Boveri's theory of fertilization rests on the identification of the centrosomes of the sperm aster with a definite formed element (centrosome) introduced into the egg by the spermatozoon; but it has never been demonstrated in any case in all the literature on the subject of fertilization that the centrosomes of the cleavage spindle, or indeed of the sperm aster, are derived from any definite formed element of the spermatozoon. Boveri himself admits this in his sixth cell study ('07, page 266); and so long as definite proof of the continuity of the so-called sperm-centrosomes from the spermatid up to the formation of the first cleavage spindle is lacking, all of Boveri's observations are open to another interpretation than the one he has given; to the interpretation, namely, that the sperm asters represent a reaction of the egg cytoplasm to a male element, or at least a foreign element, represented for the most part by the sperm nucleus.

The biological analysis of fertilization seems to me to rest now upon the problem of the origin of the sperm aster in the egg. More crucial evidence is needed on this point, and I do not believe that any refinement of cytological technique will give the result. Experimental evidence is needed; either, as Boveri ('88) suggested, the introduction of a non-nucleated spermatozoon in the egg to prove whether or not the sperm asters would arise without the nucleus and fertilize the egg, or the introduction of only the

anterior part of the sperm into the egg to prove whether or not the sperm nucleus without the centrosome, which is contained in the middle piece of the spermatozoon, would cause the production of asters in the egg-cytoplasm.

As Boveri, among others, has pointed out, there is not only one, but several stages of inhibition in the history of the egg. This may be illustrated by noting the stages at which in the eggs of various animals the need for fertilization arises. In some eggs it is before the rupture of the germinal vesicle (*e.g.*, Nereis), in others at the time of the mesophase of the first maturation spindle (*e.g.*, Chaetopterus and Cerebratulus), in others again after the formation of the first polar body (*e.g.*, Amphioxus, amphibians), in others again, not until after the formation of both polar bodies (*e.g.*, sea-urchin). There is no doubt that the last stage of inhibition is the most difficult one to overcome, both because many eggs pass by the earlier stages without apparent specific stimuli and also because it is possible to cause eggs that normally stop at the first or second stage of inhibition to pass on to the last stage by stimuli that are ineffective when this stage is reached, (*e.g.*, Nereis as noted in this paper and Chaetopterus as noted in various earlier papers).

The nature of the inhibition that causes the need for fertilization is a most fundamental problem. Is it the same in all these cases, *i.e.*, a gradually increasing inhibition that may be effective before maturation, but in some cases not until maturation has progressed a certain distance, or even until it is complete?[4] If it is the same cause at all these stages then it is certain that the need for fertilization is not due to any defect of the egg-centrosomes, for the pause takes place in Chaetopterus (e.g.) while the egg-centrosomes are at the very height of their activity. If,

[4]Bataillon ('10) holds the view that the nature of the inhibition is the same whether the arrest is at the stage of the resting nucleus or in the height of karyokinesis. He holds the view that the inhibition is due to accumulation of excretory products and that the stimulus to development is essentially a process of elimination. Bataillon's paper was received after my own was completely written. His interesting results will be considered more fully in my next paper. In Part I of the present paper (last paragraph) I have presented a view similar in some respects to Bataillon's.

therefore, we are to hold to the theory of Boveri in its literal sense, we must believe that there are different kinds of inhibition. However, it is, I believe, simpler and more logical to hold that the inhibition differs only in intensity at these various stages; and this point of view seems to be supported by the fact that the same stimulus which at a lower intensity will cause only maturation to take place in Chaetopterus, at a higher intensity will cause differentiation also to proceed, though in this case without cleavage. Boveri ('07), however, holds that there are different kinds of inhibition, that the postulated degeneracy of the egg-centrosomes after maturation is in a sense the more primitive, and that other kinds have been secondarily acquired, a point of view that gives a more or less definitely teleological aspect to the question.

From a physiological point of view we might inquire, what are the conditions that cause the postulated sudden degeneration of the egg-centrosomes? Such a condition if found, would be nearer the fundamental cause of inhibition of the egg and it might turn out to be the same cause that conditions in so many cases an earlier arrest of activities in the egg.

The experiments on artificial parthenogenesis are sometimes regarded as involving the entire problem of fertilization. But if it be true, as many believe, that biological fertilization, (if I may be pardoned such an expression) is fundamentally a sexual reaction, then the physico-chemical analysis of fertilization must compass the entire problem of sex, which is much wider than the problem of parthenogenesis. The physico-chemical analysis of fertilization has dealt, up to the present exclusively, with the latter problem, and for this reason the earlier title of such studies 'artificial parthenogenesis', seems to me much more fitting than 'chemical fertilization' which is sometimes loosely used. From the zoological point of view, at least, parthenogenesis and fertilization are not interchangeable functions. There is a factor present in fertilization which is absent in parthenogenesis, and the latter is never the exclusive mode of reproduction among animals. The biological analysis of fertilization therefore involves problems that do not occur in the physico-chemical analysis of parthenogenesis.

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PLATE 1

EXPLANATION OF FIGURES

1 Axial section of an unfertilized normal ovocyte of Nereis, fixed in Flemming's fluid, weaker solution. In this fixing fluid the yolk granules swell and tend to run together. The oil drops are dissolved out in the preparation and are represented by empty spaces. *v.m.* vitelline membrane. *c.l.* cortical layer, from which the jelly is formed.

2 Section of an egg of Nereis, fixed in Meves' fluid five minutes after insemination. The cortical layer is already somewhat reduced in thickness. The yolk granules are not swollen. The oil drops are not dissolved out. The section is approximately horizontal. *c.l.*, remains of cortical layer; *v.m.*, vitelline membrane.

3 Section of an egg of Nereis, fixed in Meves' fluid fifteen minutes after insemination. The cortical layer has entirely disappeared, and the perivitelline space is formed. The germinal vesicle is breaking down and the first maturation spindle is forming. *p.v.* perivitelline space. *v.m.*, vitelline membrane.

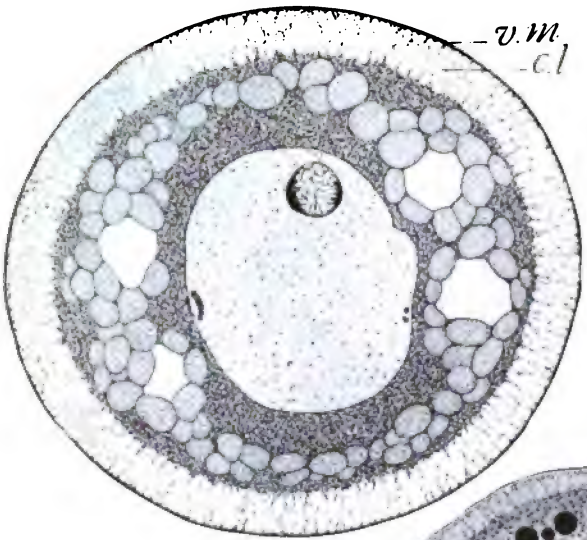
4 Section of an egg of Nereis, fixed in Meves' fluid fifty-seven minutes after insemination. Only one centrosome of the sperm amphiaster is shown.

5 Section through the first cleavage-spindle of Nereis, normal, one hour and twenty-seven minutes after insemination.

6 Tetrapolar second maturation spindle of Nereis. See text for description (p. 378). Three egg nuclei are formed in such a case.

7 Egg nucleus of egg of Nereis in which the spermatozoon was removed by centrifuging. The chromosomes of the first cleavage are formed, but there are no asters. Cf. fig. 5.

All figures drawn with the camera lucida with Zeiss comp. oc. 6 and 2 mm. hom. oil im. obj.



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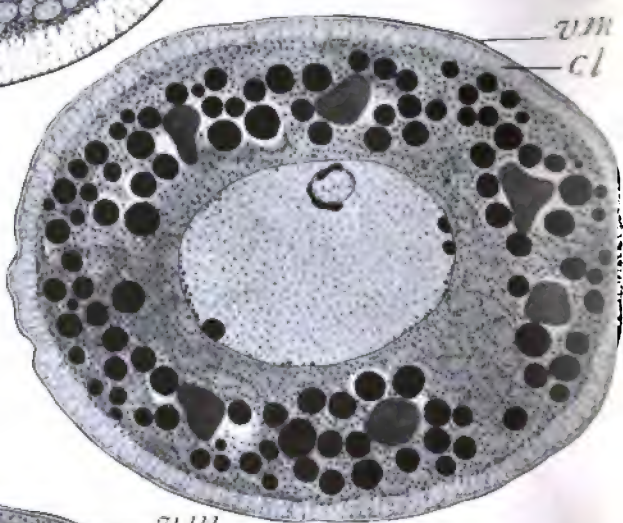

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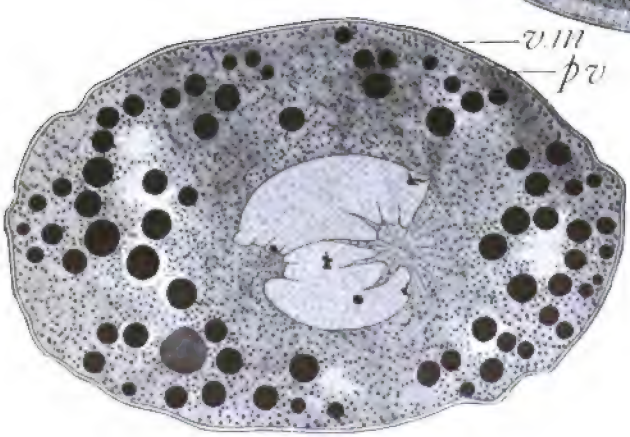

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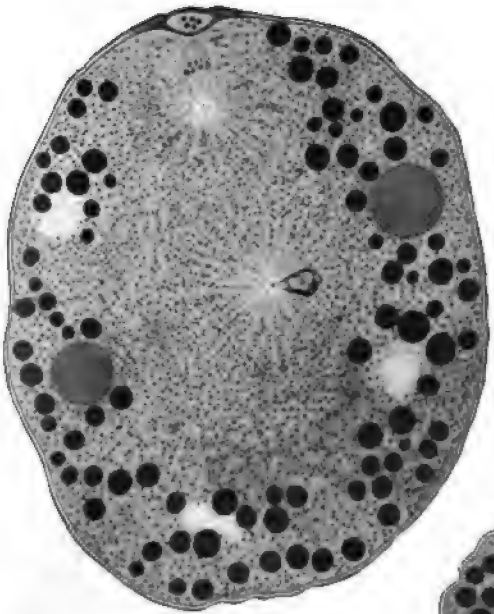

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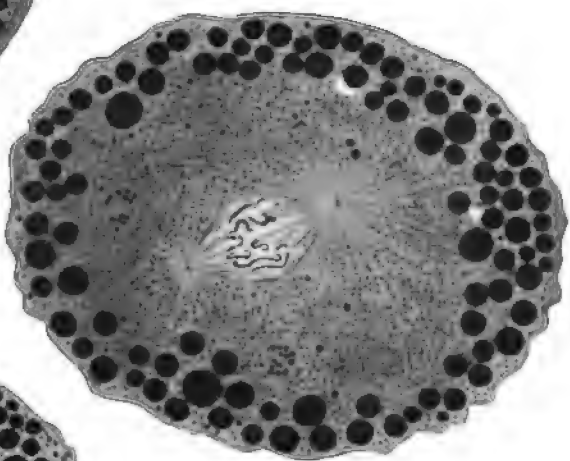

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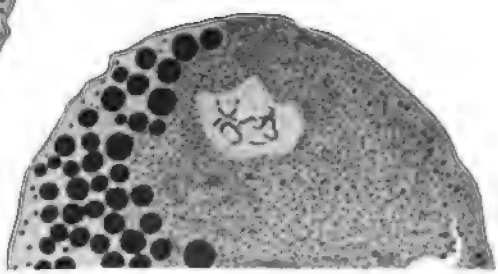

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[*Reprinted from* SCIENCE, *N. S., Vol. XXXV., No. 899, Page 471, March 22, 1912*]

The Penetration of the Spermatozoon and the Origin of the Sperm Aster in the Egg of Nereis:

FRANK R. LILLIE, University of Chicago.

(1) As described in a previous paper,[1] the egg of *Nereis* secretes a large quantity of jelly, derived from spherules of a cortical layer, immediately after contact of the spermatozoon. The perforatorium of the spermatozoon penetrates the vitelline membrane and becomes inserted in a well-marked entrance cone. *Fixation granules,* undoubtedly derived from the spermatozoon, appear at the tip of the perforatorium in the cone. The substance of the cone becomes differentiated by staining reaction and behavior to form an organ definitely concerned in the subsequent penetration of the spermatozoon. (2) The spermatozoon remains external to the egg embedded in the jelly for 45 to 50 minutes after attachment. The head is then drawn by the cone through the vitelline membrane into the egg in the form of a thick strand of chromatin several times the length of the original head of the spermatozoon. *The middle piece and tail remain external,* and may be seen on the vitelline membrane up to the time of cleavage. They never enter. (3) The chromatic thread representing the head of the spermatozoon then contracts to form a vesicular nucleus, still attached by the perforatorium to the cone. Nucleus and cone then rotate through 180° and the sperm aster arises always at the point of the nucleus farthest from the cone, thus in the position of the base of the sperm head. The problem of the origin of the sperm centrosome in the egg is considered in the next abstract. (4) The "fixation granules" are *possibly* cytoplasmic elements introduced by the spermatozoon, though they come from the apex of the spermatozoon. No other demonstrable cyto-

[1] *Jour. Morph.*, 22, 1911.

plasmic elements are introduced by the spermatozoon. (5) After origin of the aster the sperm nucleus separates from the cone, and the latter gradually disintegrates in the cytoplasm. The sperm amphiaster becomes the cleavage amphiaster.

On the Fertilising Power of Portions of the Spermatozoon: FRANK R. LILLIE.

If unsegmented eggs of *Nereis* be centrifuged about 60 revolutions of the hematocrit of the Bausch and Lomb hand centrifuge in about 40 seconds, thirty to forty minutes after insemination, the mass of eggs accumulates at the distal end of the tubes and becomes pressed together. The jelly, which is of less specific gravity than the eggs, then separates from the latter and forms a layer above the eggs. In squeezing through the narrow interstices between the closely packed eggs it rubs over the surface of each egg, and in many cases it carries the attached spermatozoon away with it. In other cases, especially if the eggs are centrifuged shortly before the time of penetration of the spermatozoon, it draws out the substance of the head of the spermatozoon, which is very ductile at this time, into a strand; and in numerous cases it carries away the tail and middle piece, or variable portions of the head in addition. Partial sperm-heads of all sizes are therefore left attached to the egg by the perforatorium. Such partial sperm-heads then penetrate, being drawn in by the entrance cone as in the case of normal fertilization, and they form partial sperm-nuclei attached to the entrance cone within the egg. Rotation then takes place and an aster invariably arises in connection with the partial sperm-nucleus at the point most distant from the cone, *i. e.*, at the most basal point of the sperm-nucleus. By using large quantities of eggs and by preserving at various stages one can study all stages of these processes in sections, and the above statements are based upon large numbers of observations. It is therefore proved (1) that the sperm centrosome is inessential for formation of the sperm aster within the egg; (2) the origin of the sperm aster is a nucleo-cytoplasmic reaction; (3) the sperm-nucleus is polarized with reference

to this reaction inasmuch as the sperm aster arises always at the most basal point of the partial sperm-nucleus, with reference to the orientation of the nucleus in the sperm head. The conclusion therefore involves the conception of a certain differentiation between egg-nucleus and sperm-nucleus, for the former has no independent capacity of initiating karyokinetic phenomena, whereas the latter has.

The two preceding papers will be published in full in the *Journal of Experimental Zoology*.

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MAY, 1912

STUDIES OF FERTILIZATION IN NEREIS

III. THE MORPHOLOGY OF THE NORMAL FERTILIZATION OF NEREIS
IV. THE FERTILIZING POWER OF PORTIONS OF THE SPERMATOZOÖN

FRANK R. LILLIE

From the Marine Biological Laboratory, Wood's Hole, Mass., and the Hull Zoological Laboratory, University of Chicago

FORTY-EIGHT FIGURES

ELEVEN PLATES

In the preceding numbers of these studies dealing with the cortical changes of the egg, and with partial fertilization, reference has been made to certain phases of the normal fertilization. A detailed study was found to be necessary to clear the way for further considerations. The results of this detailed study are presented in the first part of the present paper. The second part deals with the fertilizing power of portions of the spermatozoön and with the theory of fertilization.

III. THE MORPHOLOGY OF THE NORMAL FERTILIZATION OF NEREIS

1. The spermatozoön: (Fig. 1, a, b, c, d)

The spermatozoa are unusually large. The head terminates in a very delicate perforatorium which possesses a slight distal enlargement.[1] Between the base of the perforatorium and the chromatin of the head is some differentiated material forming a head-cap (*h. c.*). This is very distinctly differentiated in position and staining reaction from the chromatin of the head. The chromatin of the head is not so condensed as in most spermatozoa;

[1] In the living spermatoön the perforatorium is shorter and shaped more like the spike of a helmet.

it is often arranged in two masses as shown in fig. 1 with some karyolymph; sometimes the arrangement is more reticular, but the karyolymph is always in evidence in good preparations. The middle-piece is very distinctly set off from the head; it is ring-shaped, very broadly attached to the base of the head. The attachment of the tail is to the *margin* of the ring, and is therefore excentric to the axis of the head. There is a decided asymmetry, particularly illustrated in *b*, *c*, *d*, of fig. 1.

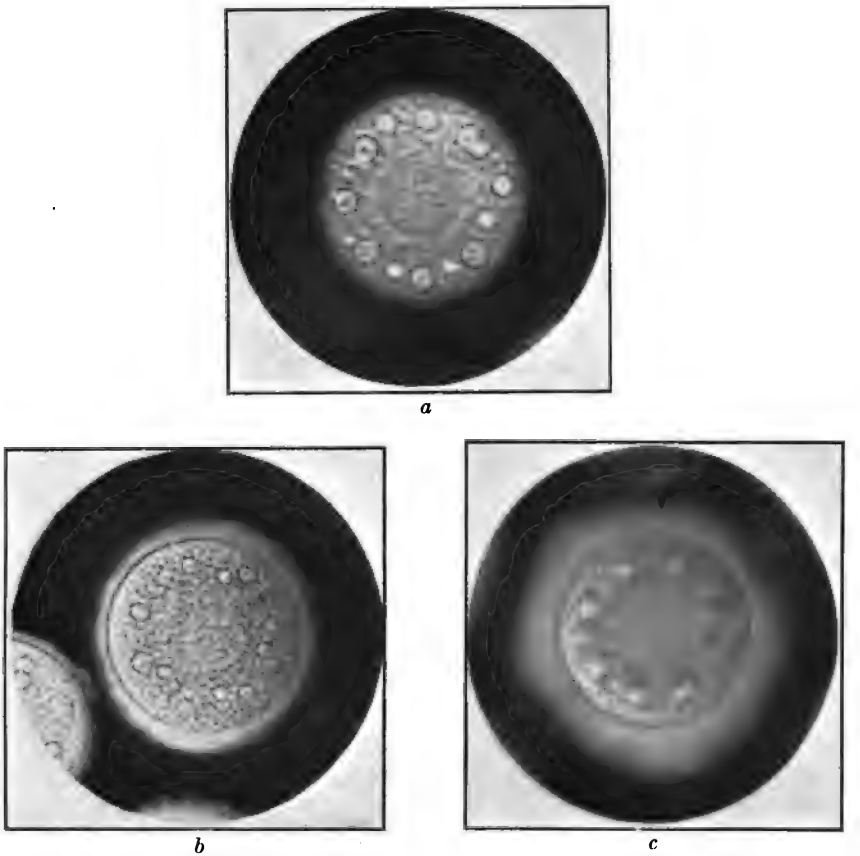
2. The ovum

The ovum has been described in the preceding parts (Lillie, '11) of this study. A brief description will therefore serve here. It is about 100μ in greatest diameter, somewhat flattened in a polar direction, and girdled by a double zone of large oil drops embedded in the yolk-bearing protoplasm (text fig. 1). It is bounded by a vitelline membrane and possesses a coarsely alveolar cortical layer about 7μ in thickness external to the yolk-bearing layer.

3. Observations on fertilization in the living egg

If the eggs remain unfertilized in the sea-water, maturation does not take place, and the egg remains unchanged with intact germinal vesicle.

a. The cortical changes. When insemination takes place a large number of spermatozoa become attached to the ovum if the sperm is present in excess. In about two to three minutes all spermatozoa, with the exception of one, which is alone concerned in the subsequent fertilization, begin to be carried away from the surface of the egg by an outflow of jelly from the ovum. This is more particularly described in the first of these studies (F. R. Lillie, '11). The jelly is formed from the alveolar contents of the cortical layer, which gradually disappears, until in the course of about fifteen minutes the original cortical layer is represented only by the perivitelline space and the delicate walls of the original alveoli crossing this space to the plasma membrane (see study 1).



Text fig. 1 Three photographs of eggs of Nereis in ink; *a*, before insemination; *b*, three minutes after insemination; *c*, twelve minutes after insemination. *a* and *b* were taken with direct sunlight and short exposure (one second), *c* with diffuse light and longer exposure (ten seconds). See text for description.

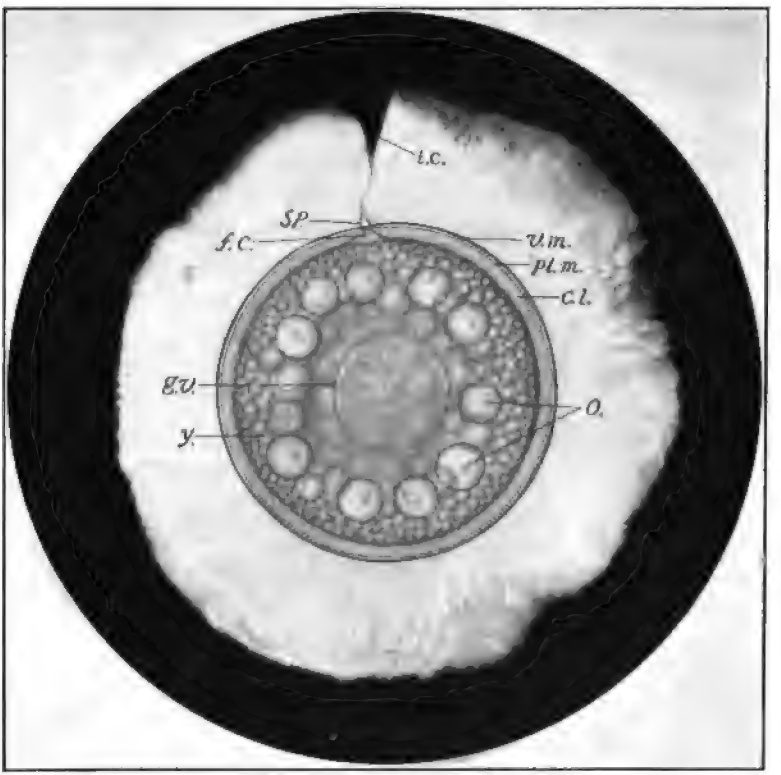
The formation of the jelly may be most readily observed if the eggs are inseminated in sea-water, into which India-ink has been ground so as to make a black suspension. I here reproduce three photographs showing the formation of the jelly under such circumstances (text fig. 1, *a*, *b*, *c*); *a* is an egg taken before insemination; the germinal vesicle and the oil globules stand out very distinctly; the cortical layer may be seen on the left side of the figure, but it does not come out very distinctly because it is seen through a layer of ink between the cover slip, which touches the

top of the egg, and the equator of the egg. Fig. 1 *b* shows the amount of jelly formed about three minutes after insemination; the cortical layer is already much reduced. Fig. 1 *c* was taken twelve minutes after insemination; it shows a great increase in amount of jelly due not only to continued secretion from the egg, but also, presumably, to swelling of the jelly already formed. The walls of the alveoli which contained the jelly-forming spherules may be seen on the right side of this photograph crossing the perivitelline space to the plasma membrane. On the upper side may be seen the fertilization cone and external to it outside the vitelline membrane the spermatozoön. A conical pointer of ink is directed to the spermatozoön. This forms regularly in eggs inseminated in ink and is due to the ink particles pressing in along the tail of the spermatozoön embedded in the jelly, owing to their Brownian movements. This ink cone remains even after the spermatozoön has penetrated and thus serves as indicator of the point of entrance of the spermatozoön up to the time of cleavage. A study of the relation of the point of entrance to the first cleavage plane by one of my students is now under way (see Just, '12).

Text fig. 2 is a drawing from the living egg based on such a photograph as 1 *c*. The details are all clearly shown here and are described in the legend of the figure.

b. The fertilization cone. The spermatozoön, that remains after the first jelly-formation, is attached by its perforatorium to the vitelline membrane. The protoplasm of the ovum immediately beneath now forms a conical elevation (fertilization cone) which crosses the perivitelline space and becomes attached to the vitelline membrane beneath the spermatozoön (text figs. 1 *c* and 2). The fertilization cone then gradually retracts and disappears, drawing the vitelline membrane with it so as to form a depression in which the spermatozoön is included. At this stage one may easily imagine that the spermatozoön has been taken into the egg, as it is apt to be concealed in the depression of the membrane; but this is not the case. The stage of greatest development of the depression, corresponding to the complete retraction of the fertilization cone, is about twenty-two to twenty-five minutes after insemination (see figs., Lillie '11, p. 369).

The fertilization cone is no longer seen in the living egg. But the sections show that its substance has become so modified as to form a definite cell-organ, which can be followed for some time after penetration of the spermatozoön.



Text fig. 2 Egg of Nereis fifteen minutes after insemination in ink. The black outer circle represents the ink, the clear area between this and the egg is the jelly; *c. l.*, cortical layer showing the radiating lines of the emptied alveoli; *f. c.*, fertilization cone; *g. v.*, germinal vesicle, drawn perhaps a little too strongly; it begins to break down about this time; *i. c.*, ink cone; *o*, oil drops; *pl. m.*, plasma membrane; *sp.*, spermatozoön; *v. m.*, vitelline membrane; *y.*, yolk spherules.

c. Penetration of the spermatozoön. Following the development of the depression in the vitelline membrane consequent upon the retraction of the fertilization cone, the perivitelline space

narrows around the entire egg, the depression in the vitelline membrane therefore disappears and the spermatozoön again becomes prominent externally. It remains external until about forty to fifty minutes from the time of insemination and then disappears rather abruptly within the egg. Its penetration coincides with the late anaphase or telophase of the first maturation division, or with the extrusion of the first polar body.

A definite granule to which the tail is attached always remains on the membrane at the point of penetration. The cytological study shows this to be the middle piece, which does not enter the egg. Not only have I repeated this observation frequently in two successive seasons, but it has been demonstrated to classes of students at Woods Hole and to some of the investigators there. *The middle piece and tail of the spermatozoön do not enter in the fertilization of the egg of Nereis.* In view of the emphasis which has recently been put on cytoplasmic contributions by the spermatozoön in fertilization, I paid particular attention to this point in the summer of 1911, and was able to determine not only that it fails to enter at the same time as the sperm-head, but that it can be demonstrated external to the membrane up to the time of the first cleavage at least (cf. also Just, '12).

I have seen the penetration of the spermatozoön take place in all positions from the animal to the vegetative pole, and I cannot say that there is any preferred point of entrance with reference to the poles. The existence of polyspermy, which is not infrequent, proves that there is no preferred meridian for penetration. So it would appear that any point on the surface of the ovum may be used for penetration.

4. The phenomena of fertilization as seen in sections

a Before penetration. Technique: The eggs are fixed for an hour in Meves' modification of Flemming's fluid made as follows: Chromic acid 0.5 per cent, 15 cc.; osmic acid, 2 per cent, 3.5 cc.; glacial acetic acid, three drops. Staining in iron haematoxylin alone.

a. The spermatozoön is readily found in sections prior to penetration, and all parts can frequently be recognized, though the tail is usually invisible owing to extraction of the stain. The middle piece is always distinctly differentiated, but it is rather variable in appearance. Its ring-form is usually distinguishable in sections and in some cases one or more minute granules may be distinguished in it. The head stains solid black in the iron haematoxylin. Fifteen minutes after insemination (fig. 2, *a*, *b*, *c*) the perforatorium has penetrated the vitelline membrane and is in contact with the fertilization cone.

The fertilization cone itself presents a rather remarkable appearance, and it deserves very careful attention, owing to the rôle that it plays in the penetration of the spermatozoön. Fifteen minutes after insemination it projects somewhat above the contour of the egg, and its substance, which is practically homogeneous, stains much more deeply in the iron haematoxylin than the neighboring cytoplasm. This difference in staining reaction so suddenly acquired indicates a modification of its substance which might be conceived as due to slight coagulation by a fluid introduced by the spermatozoön. In any event the change in stain is due to its relation to the spermatozoön, and it is the first indication of its conversion into a specialized cell-organ.

A fertilization cone has been described in various eggs (echinids, asteroidea, etc.), but in all cases previously described its significance has been, apparently, merely temporary and local, a reaction of the ovum to the spermatozoön with no definable function of its own. The case of Nereis, however, is very different, as will be seen from the following description.

At twenty-seven minutes after insemination the fertilization cone no longer forms any projection (fig. 3); it is usually, on the contrary, somewhat depressed in the center. The perforatorium stains somewhat more strongly than before.

At thirty-four minutes after insemination the perforatorium has entered the surface of the fertilization cone to a slight extent (fig. 4), and a small granule appears at its tip, surrounded by a slight clear area. A little later (fig. 5, *a*, *b*, *c*, *d*, thirty-seven minutes after insemination) there is a group of three or four black

granules, which we shall name *attachment granules*, at the tip of the perforatorium embedded near the surface of the fertilization cone. In one case (fig. 5 *a*) the perforatorium is seen to be double, a condition that I have also observed once in a preparation of spermatozoa alone. The perforatorium now stains very strongly.

The spermatozoön is now, therefore, actually anchored in the substance of the fertilization cone by the perforatorium and attachment granules noted above. I interpret the increased strength of stain in the perforatorium as due to the flow of substance through it from the spermatozoön to form the granules found at its tip in the fertilization cone.

One can give only a more or less probable interpretation of these phenomena. The extreme delicacy of the perforatorium and the presence of a distal enlargement on it make it improbable that it bores through the membrane in a merely mechanical way; and the improbability is strengthened by the fact that the spermatozoön is absolutely immobile after attachment to the membrane. It seems *probable*, therefore, that the vitelline membrane is weakened at the point of application of the perforatorium, presumably by a fluid flowing through the perforatorium, though on account of its extreme delicacy it is impossible to be certain that the perforatorium is tubular. The staining reaction and differentiation of the fertilization cone could then be explained by the assumption that it is affected by a fluid furnished by the spermatozoön, and the appearance of the granules within the fertilization cone as due to inflow from the spermatozoön through the perforatorium. The mechanism of the spermatozoön certainly appears more complex than we have hitherto suspected. I would assume, therefore, that the material of the head cap, forming a reservoir of material at the base of the perforatorium, functions in relation to penetration.

b. Penetration. The actual penetration of the head of the spermatozoön is shown in figs. 6, 7 and 8, all taken from material preserved forty-eight and one-half minutes after insemination. The complex made up of the fertilization cone and the head of the spermatozoön acts as a unit. The cone retreats into the interior of the protoplasm and the head of the spermatozoön becomes

a narrow chromatin band as it enters through the minute aperture in the vitelline membrane. Figs. 9 *a* and 9 *b* (fifty-four minutes after insemination) show penetration completed and it will be noted that the middle piece remains external.

Examining now the details of this process, every stage of which is found in the preparations, we may note: (1) The sperm nucleus enters through an aperture in the membrane much smaller than itself, and is hence drawn out into a strand. (2) The inner end of the entering sperm nucleus begins to swell, by absorption of fluid soon after its entrance has begun (figs. 6, 7, 8). (3) Striae appear in the protoplasm between the fertilization cone and the surface of the egg. (4) When penetration has actually begun (fig. 6) the distance, corresponding to the length of the perforatorium, which originally separated the chromatin of the spermatozoön from the attachment granules in the fertilization cone has become very much reduced. The sperm nucleus is now almost or actually in contact with the fertilization cone (figs. 6, 7, 8).

If we inquire into the mechanism of this process, it is quite obvious that the initiative, so to speak, is on the side of the ovum. It is inconceivable that the spermatozoön should be the primum movens and push the fertilization cone before it into the protoplasm without being coiled up or bent within the protoplasm. But, in the many instances observed, it is invariably as straight as shown in the figures. The fertilization cone indeed *appears* to actively penetrate, or to be engulfed by the egg protoplasm, and to draw the sperm nucleus after it through the narrow aperture in the vitelline membrane.

The final stage of penetration is shown in figs. 9 *a* and 9 *b*. The entire sperm nucleus is now within the egg cytoplasm, some distance from the periphery, and it will be seen that the middle piece has remained without on the egg membrane. This is perfectly characteristic, if not invariable, and the same observation was made repeatedly on the living egg as already stated. The picture is perfectly clear as shown under a very high magnification in fig. 9 *b*, and not only is the middle piece plainly external, but there is no sign whatever of any sperm-component at the base

of the sperm head. An assumption that the spermatozoön introduces any differentiated structure at the base of the head could, in this case, be due only to a preconception in its favor.

c. Revolution of the sperm-nucleus and origin of the sperm-aster. The original orientation of the sperm nucleus within the egg is perfectly apparent, owing to the association of its apex with the fertilization cone from the very beginning of penetration. The whole complex of fertilization cone and sperm nucleus now rotates 180 degrees, so that the fertilization cone, which was placed centrally to the sperm nucleus, comes to lie peripheral to it. In fig. 10 *a* (fifty-four minutes after insemination) the revolution is shown nearly completed. Fig. 10 *b* shows the cone-nucleus-aster complex of the same egg more highly magnified; and figs. 10 *c* and 10 *d* show the entrance point of the spermatozoön involved, with tail (10 *c*) and middle piece (10 *d*) external to the vitelline membrane. The sperm aster is beginning to form in this stage opposite to the fertilization cone, thus in the position of the original middle piece, which, as we have seen, was left on the egg membrane. A minute granule, actually in the substance of the nucleus (fig. 10 *b*) is the point of focus of the rays. In fig. 11 (sixty-four minutes after insemination) this granule, now more clearly recognizable, is still in contact with the sperm nucleus. The rotation of the sperm nucleus is invariable, and no exception has been seen in the numerous cases observed; the point of origin of the sperm aster is just as invariable.

The fact that the sperm aster arises from the base of the sperm nucleus as it is oriented in the spermatozoön, not only in Nereis but in all other forms accurately determined, is unquestionably of fundamental significance. I may, however, be permitted to point out that the usual interpretation, which connects the sperm aster with the centrosome of the spermatid (occupying this position in the spermatozoön) is not the only possible interpretation. It is important to emphasize this point because such an interpretation ascribes the fertilizing power of the spermatozoön in the last analysis, not to the spermatozoön as a whole, but to a minute part of it, the centrosome; and it involves a whole theory not only of fertilization, but of cell division in general, and much of cellular

physiology, by ascribing certain very general functions and powers to this minute cell element.

The alternative interpretation, which I would present, is that the appearance of the sperm aster in a position definitely oriented with reference to the sperm nucleus may be a nucleo-plasmic reaction localized by polarity of the nucleus.

In any case, if the centrosome theory is to be retained for Nereis, it becomes necessary to assume that, in this form, the centrosome is contained, not in the middle piece, but in the nucleus of the spermatozoön. In the experimental study, which forms the second part of the present paper and study number 4 of the series, I shall present crucial evidence against this assumption; discussion of the matter may therefore be postponed.

After the appearance of the sperm aster, the nucleus penetrates yet more deeply within the egg and leaves the fertilization cone behind near the inner margin of the yolk (fig. 11), where it soon disappears. So far as I know, it is a perfectly unique cell organ, except in its earliest stages, and it is certainly surprising to find it in so highly differentiated a condition, and with apparently so active a function in the penetration of the spermatozoön, as it is in Nereis.

d. Division of the sperm aster. The sperm nucleus moves towards the center of the egg, and the aster separates from it a little and divides, forming an amphiaster. A secondary spindle then arises between one or both of the sperm-centrosomes and the inner centrosome of the second maturation spindle (figs. 12 and 13). This has been very fully described by Bonnevie ('10). Shortly after the sperm amphiaster is formed, it is noticeable that one of the asters and centrosomes is decidedly larger than the other (figs. 13 and 14 *a*). Bonnevie has also called attention to this fact, but has not, as I believe, sufficiently emphasized the fact that it is a secondary condition. I must dissent a little from her view that the sperm aster does not always divide prior to the completion of the second maturation division; in my material, at least, the amphiaster is invariably formed prior to this time.

e. The germ nuclei and origin of the cleavage centers. After the second polar body is formed, the egg aster gradually dwindles and

disappears (figs. 14 *a*, 14 *b* and 15). There can be not the least doubt of this fact in my material, though Bonnevie, working on the same form, but with different cytological methods, remains doubtful on this point. I shall not attempt an elaborate cytological analysis of the matter here (though the evidence from this side is, I believe, conclusive), because in eggs from which the sperm nucleus has been removed experimentally the gradual disappearance of the egg aster can be traced with complete certainty, owing to the absence of any possible confusion with the sperm aster (see second part of this paper, p. 440). The fully formed egg nucleus in such eggs never has a trace of the aster (see studies 2 and 4). The chromosomes of the egg swell and form vesicles (figs. 14 *a*, 14 *b*, 15), which gradually fuse together and establish the egg nucleus. At the same time the sperm nucleus begins to enlarge (fig. 14 *b*) and the sperm amphiaster becomes less distinct than before and its radiations less extensive (figs. 13, 14 and 15). The sperm amphiaster continues to wane and when the two germ nuclei have come together, the smaller aster has become indistinguishable (fig. 15). The larger aster can, however, always be distinguished through the stages of the germ nuclei, and can be seen to become the larger aster of the first cleavage spindle after the partition wall between the germ nuclei has disappeared (figs. 16 *a*, 16 *b*, 17). At this time a much smaller aster arises opposite the larger (fig. 17), like it in the plane of apposition of the germ nuclei. The first cleavage spindle is thus heterodynamic from its inception, and the first cleavage of the egg is strikingly unequal, as is well known from Wilson's study (Wilson, '94).

In the preceding paragraph I have summarized a very complicated period of the fertilization process to which I have given much study, and which exhibits many interesting cytological details, as for instance, the accumulation of granules in the neighborhood of the germ nuclei (figs. 15 and 16 *a*) which almost certainly escape from the latter (fig. 16 *a*). As regards the main point, the origin of the cleavage centers, there can be no doubt that the larger one is derived from the larger aster of the sperm amphiaster, for it can be followed continuously and is

never absent. The simplest interpretation of the smaller cleavage center is that it represents the smaller sperm aster, although there is a brief period when it is not demonstrable, owing (possibly) to defect in the cytological technique. It is my opinion, then, that the sperm amphiaster becomes the cleavage amphiaster in Nereis, as in so many other animals.

5. Discussion

If any apology is needed for presenting so strictly a morphological study of such an apparently threadbare subject as the fertilization of the ovum, I might say that the impulse to make it came from an experimental study, and that it is necessary to the experimental results which follow. It has, moreover, yielded some details of observation which deserve to go on record on their own account.

The conclusion that the cleavage centers arise from the sperm centers is in agreement with many other studies. But I am unable to accept the usual conclusion that the sperm centers arise around a centrosome introduced by the spermatozoön into the egg, and that, therefore, the sperm centrosome is the fertilizing agent of the spermatozoön, and the sperm nucleus concerned exclusively with amphimixis. The crux of the problem is precisely here on the question of the origin of the sperm centers. The fact that the middle piece of the spermatozoön which usually includes the spermatid centrosome does not penetrate the egg in Nereis is evidence of a certain amount of value only. Defects in cytological technique may always be invoked to explain failure to observe the introduction of a centrosome by the spermatozoön. Little as I may be inclined to admit this, it is necessary to grant some force to this objection where such delicate cytological details are involved. It would, however, I believe, be recognized as crucial evidence that the sperm centrosome is not necessary to fertilization, if a distal fraction of the sperm head alone were proved to form a sperm aster, a certain portion of the base of the sperm head as well as the middle piece being prevented from entering. Such results are described in the second part of this paper.

There has been much discussion during the past year concerning a cytoplasmic basis of certain aspects of inheritance, and a consequent re-investigation of the penetration of the spermatozoön. Following Meves' study ('11) of the rôle of plastochondria in the fertilization of Ascaris, in which he concluded that "the plastosomes represent the hereditary substance of the cytoplasm as the chromatin does that of the nucleus," a number of authors investigated the penetration of the spermatozoön in echinids. Dantan ('11) asserts that in Paracentrotus lividus and Psammechinus miliaris, the entire spermatozoön enters the egg, and he concludes that fertilization should be defined as the union of two complete gametes which fuse nucleus to nucleus and cytoplasm to cytoplasm. Witschi ('11) describes a case in Strongylocentrotus in which the tail of the spermatozoön entered in fertilization, but he thinks it probable that in this form the tail is oftener left on the membrane. Ries ('11) describes a curious shedding of an involucre in the penetration of the spermatozoön in Strongylocentrotus, but he believes that the axial structures of head, middle piece and tail enter. His account must, however, be accepted with considerable reserve until confirmed. Finally, Meves ('11*a*) has studied the relation of the middle piece in the fertilization of Parechinus miliaris, and believes as the result of his observations that it probably furnishes plastochondria. He says nothing about penetration of the tail, so it is fair to assume that it does not occur in Parechinus.

The classical accounts of the penetration of the spermatozoön in sea-urchins, according to which the tail is left on the membrane and only head and middle piece enter seem to be on the whole confirmed so far as the main principle (i.e., the non-essential character of the tail in fertilization) is concerned, since both entrance and non-entrance have been observed. The tail cannot therefore be regarded as supplying a cytoplasmic basis for inheritance in sea-urchins.

I have shown that in Nereis the middle piece of the spermatozoön is likewise left on the membrane, so we cannot look to it as a cytoplasmic basis for inheritance in this form as Meves does in the sea-urchin. On the other hand it is possible that the fixation

granules introduced by the spermatozoön represent a cytoplasmic element (whether concerned in inheritance or not), but of this we cannot be certain until their derivation is better known.

The only characteristic thing about the cytoplasmic elements introduced by the spermatozoön is their great variability as to quantity and character in different animals. In Ascaris a very large quantity of cytoplasm containing characteristic plastosomes is introduced, as Meves has shown. In many, probably most, forms with flagellated spermatozoa, the entire spermatozoön enters; in some echinids the tail is left without, and in Nereis both tail and middle piece fail to enter; and turning to plants, in phanerogams apparently nothing but the nucleus is eventually concerned. There is nothing on the cytoplasmic side to correspond with the regularity of the nuclear phenomena in both animals and plants. In such precise phenomena as those of inheritance a mechanism of equal precision is to be expected, and it must be admitted that on the cytoplasmic side no such mechanism has been discovered. Moreover, as the laws of inheritance are the same for animals and plants, a similar mechanism must exist for both, and such has been discovered only in the nuclei of the gametes. There is bad logic in the assumption that whatever parts of the spermatozoön enter the egg are necessarily concerned in the mechanism of transmission in inheritance, and the view that the cytoplasmic elements of the male gamete are concerned primarily in accessory functions of fertilization, such as locomotion and penetration, is still logically well founded.

IV. THE FERTILIZING POWER OF PORTIONS OF THE SPERMATOZOÖN

1. Introduction and methods

In the second of these studies (F. R. Lillie, '11) it was shown that the stimulus of the spermatozoön in fertilization involves two phases: (1) an external phase, prior to entrance of the spermatozoön but after its attachment to the egg, in which certain cortical changes are induced, jelly is secreted by the egg and the mechanism of maturation of the ovum is released; and (2) an internal phase beginning after the entrance of the spermatozoön,

which is necessary if cleavage of the ovum is to take place. The present study is a contribution to the analysis of the latter phase based on observations concerning the fertilizing power of portions of the spermatozoön.

It is questionable whether any direct and universal method for such an experiment could be devised, for one would have to overcome the difficulties of isolating a spermatozoön, of operating on it, and of ensuring the entrance of a part into the ovum, under precautions that would preclude the possibility of fertilization by an intact spermatozoön. These difficulties might be overcome by an instrument sufficiently delicate to enable one to amputate parts of the attached spermatozoön before its entrance into the egg. The same result has been obtained in Nereis by a method that enables one to operate in bulk, to remove fractions of the attached sperm head of varying size, to observe the entrance of the part remaining attached to the egg, and to study its fertilizing effect, at least to a certain extent.

In brief, the method consists in centrifuging the inseminated eggs of Nereis at five minute intervals before penetration and preserving the centrifuged eggs at appropriate times. The effectiveness of the method depends on conditions already described (see part 1 of the present paper, study 3 of the series) which may be summarized briefly as follows: The spermatozoön remains external to the vitelline membrane with its perforatorium embedded in the entrance cone for about fifty minutes, more or less, depending on the temperature, after insemination. It is embedded in the thick viscous jelly secreted by the egg (text figs. 1 and 2). If, now, a quantity of eggs be centrifuged with sufficient force, they first accumulate at the distal ends of the tubes in a mass which becomes closely packed together. The jelly, which is of less specific gravity than the eggs, then separates from the latter and forms a layer above the eggs. In squeezing through the narrow interstices between the closely packed eggs the jelly rubs over the surface of each egg and in many cases carries the attached spermatozoön away with it, leaving, however, the perforatorium and attachment granules in the cone as evidence of its former presence. In other cases, especially if the eggs be centrifuged shortly before the time of penetration of the spermatozoön, it

draws out the substance of the head of the spermatozoön, which is very ductile at this time, into a strand, and in numerous cases it carries away the tail and middle piece or variable portions of the head in addition. Partial sperm heads of all sizes are therefore left attached to the egg by the perforatorium. Such partial sperm heads then penetrate, if the eggs be left to develop in sea-water, and their behavior may be studied.

In each experiment 8 or 9 stages in the process of fertilization were centrifuged at five minute intervals, in order to be sure that the entire period of penetration of the spermatozoön was covered, because particularly striking results were to be expected from removal of the external part of the spermatozoön after a certain amount had entered (figs. 6 to 8). Several hundred eggs were centrifuged each time. One has to be sure that neither too little or too much centrifuging is done, and it was only as a result of considerable experience extending over three years that sixty revolutions of the handle of the centrifuge in about forty seconds, giving 7200 revolutions of the tubes at a radius of 6 cm., was selected as most favorable.

The following protocol of an experiment will show exactly how the experiments are carried out and the material secured for examination.

EXPERIMENT 16

September 21, 1911

To preserve a series immediately after centrifuging and thirty minutes later
Eggs fertilized at 8:28 A.M. Temperature of air, 18°C.; of water, 18°C.

DESIGNATION	CENTRIFUGING		PRESERVATION				OBSERVATIONS ON PER CENT OF SEGMENTED EGGS AMONG THOSE REMAINING	
		a.m.		*a.m.*		*a.m.*		*a. m.*
16.1	× 60	8:58	16.1.1	8:59	16.1.2	9:29	35 per cent	10.47
16.2	× 60	9:03	16.2.1	9:04	16.2.2	9:34	15 per cent	10.48
16.3	× 60	9:08	16.3.1	9:09	16.3.2	9:39	10 per cent	10.52
16.4	× 60	9:13	16.4.1	9:14	16.4.2	9:42	5 per cent	10.54
16.5	× 60	9:18	16.5.1	9:19	16.5.2	9:49	25–30 per cent	10.55
16.6	× 60	9:23	16.6.1	9:24	16.6.2	9:53	65 per cent	10.56
16.7	× 60	9:28	16.7.1	9:29	16.7.2	9:58	75 per cent	10.57
16.8	× 60	9:33	16.8.1	9:34.5	16.8.2	11:03	85 per cent	11.02
16.9	× 60	9:38	16.9.1	9:39.5	16.9.2	11:09.5	90+ per cent	11.03
Controls—preserved at 8:57, 9:12.5, 9:35 A.M.							90+ per cent	11.05

The eggs preserved immediately after centrifuging enable one to study the immediate effects of the separation of the jelly on the spermatozoön, and those preserved later show what portions of spermatozoa remaining attached after centrifuging have entered the egg and what their fertilizing power has been up to the time of preservation. Some of the eggs were kept living for estimation of the per cent of eggs that undergo segmentation (last column). In other experiments the eggs were preserved at different periods following centrifuging, because no single experiment gives a sufficient quantity of material for preserving a complete series of stages. In this way from a considerable number of experiments a very complete set of stages was secured.

Each lot of eggs preserved included several hundred which were embedded together in paraffine and cut in serial sections which would usually cover four or five slides under a 50 by 25 mm. cover, if all the material were cut. On one such slide I estimated by counting that there were 374 eggs present; and the 1911 material alone made 376 slides. Only about a fourth of the slides contain the desired stages, and the figures are given only to show that a large quantity of material has actually been under review to give the results. However, I may say that figs. 18 to 23 are all from a single slide, and other interesting stages occurred on the same slide; it is possible in fact to demonstrate the whole set of phenomena from a few slides of the large number prepared.

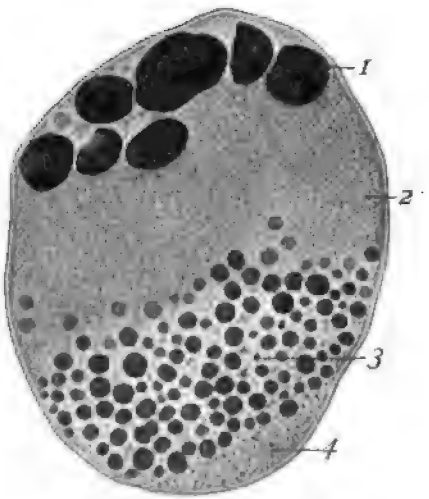
The clew to the whole set of phenomena was given by the discovery of very minute sperm nuclei in the material used for the second of these studies (F. R. Lillie, '11). In the effort to discover the origin of these minute, and presumably partial, sperm nuclei, the whole history was gradually worked out as given here.

The centrifugal force employed causes a very complete segregation of the oil and granules of the egg into four zones which are illustrated in text fig. 3, taken from a section fixed in Meves' modification of Flemming's fluid and stained in iron haematoxylin. These zones are, beginning with the constituents of least specific gravity; (1) the zone of the oil drops; (2) the hyaline zone in which the smaller basophile granules gather for the most part; (3) the zone of the yolk-spheres; (4) a zone consisting of small hyaline

spheres not stained by the osmic acid or haematoxylin, interspersed with basophile granules larger than those of zone 2. The penetration and rotation phenomena appear in the hyaline zone even more clearly than in the normal egg, hence most of the figures illustrating these phenomena are taken from the hyaline zone.

2. The effects on the spermatozoa

a. Before the beginning of penetration. The effects of the removal of the jelly on the spermatozoön have been studied especially on stages shortly before penetration begins, both because these are the stages most affected as shown by the effects on cleavage



Text fig. 3 The zones of the centrifuged egg of Nereis

(stages 16.3 and 16.4, in the preceding table) and also because spermatozoa injured at this time would presumably have a better chance to enter, being so near to the actual time of penetration.

Figs. 18 to 23 show successive degrees of injury to spermatozoa from a single experiment (6.5 of 1911). The eggs were preserved immediately after centrifuging 7200 revolutions at a radius of 6 cm. in thirty-five seconds, fifty minutes after insemination, on June 19, 1911, when the temperature of the water was still quite low and the processes correspondingly slow. In fig. 18 the entire

head of the spermatozoön is present as shown by the middle piece, but it has been drawn out to a band and shows its granular structure. Fig. 19 shows a case in which the middle piece and a small part of the base of the sperm head has been entirely removed. Figs. 20, 21, 22 and 23 show the removal of increasingly large portions of the sperm head. Cases could be illustrated beyond either end of this series in which on the one hand the spermatozoön is entirely uninjured, and on the other hand even the perforatorium is pulled out of the entrance cone. The cases illustrated are merely selections from a very much larger set of observations.

It is shown, therefore, that practically any degree of injury to the spermatozoön may be produced prior to its entrance. Cases in which the delicate perforatorium is broken next the head leaving only the cone and attachment granules are the most common as is to be expected; but the other classes of injury bear witness to the tenacity of the hold of the spermatozoön on the egg.

b. Injuries to the spermatozoön after penetration has begun. This class of injuries is relatively rare because the actual process of penetration requires only about two minutes, and the chances of involving it are therefore correspondingly few. However, I have found a considerable number of such cases in the preparations. At first I looked to such injuries as the only source of the partial sperm nuclei already observed in the egg, but as I made the observations described in the preceding section, I realized that they were not the only or indeed the chief source of such partial sperm nuclei. Figs 24 and 25 illustrate two cases from the same experiment (6.5.1, 1911) from which figs. 18 to 23 are taken. In fig. 24 it will be seen that penetration has already begun (cf. fig. 6), and the external part of the spermatozoön is ravelled out and the middle piece, at least, lost. Fig. 25 is a little more complicated; in this case penetration had probably reached a condition intermediate between figs. 6 and 7. The centrifugal force has doubled up the part of the sperm in the egg, and drawn out the external part removing the middle piece. Fig. 26, finally, is a clear cut case, the external part alone being removed entirely, and the remainder showing a clear penetration picture. This is the prob-

able interpretation of this picture, although it was taken from a preparation killed fifteen minutes after centrifuging; further penetration following centrifuging was probably prevented in this case by the large oil drops which were driven around the entering sperm.

3. Penetration of injured spermatozoa

Injured or partial spermatozoa may enter the egg, demonstrating that penetration of the spermatozoön, after attachment is once secured, is an active function of the egg and not at all of movements of the sperm itself, if any further evidence is needed on this point. But if the entire spermatozoön be removed, the cone remains superficial and does not penetrate; at least I have repeatedly found it in a superficial position fifteen to thirty or more minutes after the normal time of penetration, and I have never found it actually penetrated unless accompanied by all or a part of the sperm nucleus.

The evidence for the entrance of injured or partial sperm heads is furnished by cytological study of eggs fixed at definite periods after centrifuging. Particularly clear evidence is furnished by one series of nine stages fixed fifteen minutes after centrifuging. In the fifth stage of this series centrifuged fifty minutes after insemination and fixed fifteen minutes later, early stages of penetration are very abundant, and among them are some that show unequivocal evidence of being partial sperm nuclei, for there are numerous cases in which an injured part of the sperm head is left outside on the membrane and thus guarantees the partial nature of the sperm nucleus within. Some such cases are illustrated in figs. 27 to 32.

Fig. 27 shows a case killed fifteen minutes after centrifuging in which the connection between the internal and external parts of the spermatozoön is entirely broken. The partial sperm nucleus within the egg and the cone have already begun to rotate. In fig. 28 we have a similar case, except that there still remains a delicate connection between internal and external parts of the spermatozoön, and this condition would lead, by rupture of this connection, to the condition shown in fig. 27. It would seem,

then, that the spermatozoön may be so injured in the process of centrifuging that it ruptures in the process of penetration, and we thus learn that partial sperm nuclei may come from spermatozoa merely injured and not actually broken in the process of centrifuging. It should also be noted that in each of these cases the internal part of the spermatozoön is divided in two parts; presumably after penetration, by constriction around an injury. Fig. 29 shows a condition similar in many respects to figs. 27 and 28, in that the entire spermatozoön is present, and a large external part is separating from a smaller internal part. Figs. 30 and 31 show the early penetration of parts of spermatozoa, recognizable as such merely by their small size in the absence of the external part. The cases illustrated in figs. 30 and 31 are such as would be derived from injuries similar to those shown in figs. 20 and 21.

Rotation of the sperm cone complex has already begun in figs. 27 to 31, while the cone is much nearer the surface of the egg than in normal fertilization. Rotation begins immediately after penetration is completed, and hence takes place nearer to the surface when portions only of the sperm are concerned than when the whole sperm is concerned. In this connection the reader should compare figs. 9 and 10.

4. Stages of rotation of the partial sperm nuclei and origin of the sperm aster

Convincing evidence of the partial nature of sperm nuclei in later stages of rotation and origin of the aster is difficult to secure, in spite of the fact that the control consisting of part of the same lot of eggs preserved immediately after centrifuging may show numerous instances of partial sperm heads like those illustrated in figs. 18 to 26. The difficulty of securing unequivocal evidence arises from the fact that mere size, except in extreme cases, is no longer a safe guide; in the first place the diameters of the nuclei vary only as the cube root of their volumes, hence considerable differences in volume may be represented by undetectable differences of diameter. In the second place the sperm nuclei are not round and all are not in favorable positions for comparable meas-

urements. In the third place the volume of the sperm nuc eus normally increases considerably for some time after entrance, and the difficulty of deciding whether comparable stages are involved is sometimes great.

In spite of these difficulties, however, I have found sperm nuclei which must be interpreted as partial on the basis of their size alone, especially in later stages.

Fortunately however, it is not necessary to rely on size differences alone for in some of these stages, as in the case of the penetration stages just considered, a remnant of the sperm head may be found adhering to the membrane at the point of entrance, guaranteeing the partial nature of the sperm nucleus within. A few of these stages may be considered first:

Fig. 32 shows a case immediately continuing those described in Section 3. Here the rotation had already begun, as evidenced by the position of the cone, a very delicate connection still remaining with the external part of the sperm head impedes the rotation of the sperm nucleus within. Fig. 33 *a* and *b* shows a more advanced case; in fig. 33 *a* the cone and sperm nucleus are shown almost half rotated. One would not be able to decide from the size that it was a partial sperm nucleus, but the next section, shown in 33 *b* contains a considerable portion of the sperm head still connected with the middle piece which has remained without on the egg membrane. This is a very critical case, demonstrating that a partial sperm nucleus will rotate like a complete one. The portion in 33 *b* is entirely disconnected from the nucleus shown in 33 *a*; they are in the very act of separation. In fig. 35 we have a very fortunate section in which a completely rotated fertilization complex: cone, sperm nucleus and aster, is present, and a considerable portion of the same sperm head is found on the membrane outside the egg; the external portion is entirely comparable to the condition shown in figs. 19, 24 and 25 and there can be no question about its interpretation. Undoubtedly, the condition came from one essentially similar to that shown in fig. 25; it is, in fact, exactly what one would predict a later stage of the condition of fig. 25 to be, assuming a break to occur between the parts within and without the membrane.

Fig. 36 shows a case in which part of a sperm head has been left behind in the peripheral protoplasm at the point of entrance, and we have the completely rotated complex of cone, sperm nucleus and aster within. Another similar case is illustrated in fig. 37, and still other illustrations could be given.

It will be noted that there are very considerable size differences in the sperm nuclei of figs. 35 to 37, and all of these are distinctly smaller than the normal comparable stage shown in fig. 11. But in these cases it is not necessary to rely on the distinctly smaller size as evidence of their partial nature, for we have the evidence of sperm remnants left at the point of penetration. In figs. 38 and especially 39, however, we have cases in which the undeniably minute size of the sperm nuclei alone is sufficient evidence, taken in connection with the records of injury to the external sperm head by removal of the jelly illustrated in figs. 18 to 26, to prove that they have been derived from a mere fragment of an entire sperm head probably (in the case of fig. 39) not exceeding one-sixth to one-eighth of the bulk of the entire head, although the external part was entirely lost in this case.

It is, perhaps, not necessary, but it may be well to emphasize the fact that the cases selected for illustration are all from complete series of sections, and that the neighboring sections were always consulted for possible parts of the sperm nucleus. *The small size is not due to division by the microtome knife.* The cases described are selections from a much greater number of records.

It will be noted that if small fragments of a sperm head can produce aster formation in the egg, the possibility of polyspermy with parts of a single spermatozoön is given. This condition, which I anticipated on theoretical grounds, was finally found and is illustrated in fig. 40. There are two sperm nuclei, each with an aster, associated with a single cone. The small size of these nuclei marks them as partial, and their connection with a single cone as parts of a single spermatozoön. The only alternative explanation of this figure would be that two spermatozoa had become implanted so close together as to produce a single cone, and that they had received comparable injuries in the process of centrifuging. Against this explanation are the results of many

observations of polyspermy in none of which was implantation nearly close enough together to produce a single cone; in one series that I possess many eggs have thirty or more spermatozoa implanted, but the points of insertion are always separate. Moreover, in such a case one would expect the cone to be larger than usual, but in this case it is a little below the average size; one would also expect to find two equal groups of implantation granules with separate attachments of the nuclei, but only one group and one attachment is found here, viz., in the nucleus to the left; the granule near the right nucleus is too small to represent a separate implantation group, and moreover, it has no connection with the neighboring nucleus. Finally, it will be seen that conditions such as those illustrated in figs. 27 and 28 must inevitably lead to the condition of fig. 40 if the fragments of the sperm head do not reunite.

There is thus every reason for interpreting these two nuclei as parts of a single one. This rare find simply emphasizes the conclusions already reached concerning the fertilizing power of portions of the sperm head.

Without attempting at this place to discuss the results fully, I would, nevertheless, emphasize the two facts of greatest importance already brought out. In the first place it is shown that an apical fragment of the sperm head is able to produce an accompanying aster in the egg cytoplasm; *the sperm aster has therefore no necessary relation to the middle piece of the spermatozoön, or to the centrosome of the spermatid.* In other words, using the formation of a sperm aster as criterion, the fertilizing power of the spermatozoön is not localized in the middle piece, as supposed by Boveri and others, but is a function of even small fragments of the sperm nucleus alone. In the second place the great beauty of this material is that the orientation of the sperm nucleus, whether entire or partial, is preserved until after the origin of the sperm aster, and this enables one to determine that the sperm aster always arises in relation to the most basal point of the sperm nucleus. Altogether, I have observed well over one hundred cases of entire and partial sperm nuclei in these stages, and have never found any exception to the rule that the sperm aster arises

at the point of the sperm nucleus farthest from the cone. In other words, *the position of origin of the sperm aster is a function of polarity of the sperm nucleus*, and it is this which explains its invariable origin, so far as has been recorded in the literature, in the position of the middle piece of the spermatozoön; and the theory that a centrosome introduced by the spermatozoön is necessary for such formation is therefore shown to be incorrect.

The results so far show that sperm fragments, even of very minute size, may enter the egg in conjunction with the cone, rotate in the normal manner and produce an aster in the egg-cytoplasm. The question now arises, what is the ultimate fate of such fragments? Is their fertilizing power adequate to produce segmentation of the egg?

5. The later history of the partial sperm nuclei

Partial sperm nuclei separate from the cone and penetrate towards the center of the egg like normal ones (figs. 38 and 39). The sperm aster divides and forms an amphiaster in the stage of the anaphase of the second maturation division characterized by inequality of the two poles as in the normal. But apparently the size of the centrosomes and the extent of the astral radiations are directly proportional to the mass of the sperm nucleus concerned. This is brought out very well in fig. 41, which is a reconstruction from three successive sections showing two sperm nuclei of unequal size in the same egg. The egg in question had been centrifuged forty-four minutes after insemination and was preserved forty-seven minutes later in the stage of the telophase of the second maturation division. It will be observed that the larger nucleus (left) is accompanied by a larger centrosome and aster than the smaller one (right), and it should be stated that the aster in each case is the larger one of an amphiaster. Here, where direct comparison between nuclei of unequal size and their accompanying asters within the same egg is possible, the proportional size of asters to nuclei is obvious. I do not mean of course to assert that the proportions are mathematically accurate for this would be impossible to determine.

In general, larger sperm nuclei are accompanied by larger asters and smaller nuclei by smaller, roughly proportional, asters, after they are fully formed. Exceptions to this rule are certainly rare. I have, however, found a very few cases, two or three in all, in which a very small sperm nucleus is accompanied by a disproportionately large aster. The explanation of such cases is uncertain, but I am inclined to attribute it to a secondary reduction of the sperm nucleus after penetration and aster formation, such as might conceivably result from some form of injury received in centrifuging.

The significance of this proportional relation is at once apparent; if the aster is a product of a nucleo-cytoplasmic reaction of some kind, as we have already seen reason to believe, there must be a quantitative relation between the product (aster) on the one hand, and the reacting elements (nucleus and cytoplasm) on the other, and this is what we find.

After maturation is completed and the germ nuclei are formed, we have to find a new criterion for partial sperm nuclei. Comparison of size of the egg and the sperm nucleus alone is not very satisfactory, because both nuclei are swelling very rapidly at this time and they may meet and begin to fuse before their enlargement is complete, so that complete identity in size of egg and sperm nucleus prior to fusion is not invariable in the normal fertilization. But a valid criterion may be found in the following phenomena: the germ nuclei are formed by chromosomal vesicles, one for each chromosome, and in each vesicle a sharply marked chromatic nucleolus arises before the separate vesicles fuse. Fusion begins very early and growth of the nucleoli accompanies it; however, an elimination or dissolution of the nucleoli begins before fusion is complete, so that their number is rapidly reduced again, and they entirely disappear before the actual prophases of the first cleavage spindle. Fusion of the two germ nuclei with one another may also begin before the fusion of the chromosomal vesicles in each is complete. Under normal conditions the number of the chromatic nucleoli is probably the same in each germ nucleus in the early stages. I therefore looked for cases of striking disparity in number between the chromatic nucleoli of

the egg and sperm nuclei. Some such cases were found, but they were much fewer in number than expected on the basis of the number of partial sperm nuclei found in earlier stages. I was therefore led to suspect that the smallest sperm nuclei might disintegrate prior to this time. However, I have failed to find direct evidence for this. It may be, therefore, that the failure to find the expected number of partial sperm nuclei in the stage in question is due to the fact that the critical period for such determination is of very brief duration.

Fig. 42 illustrates a case of disparity between the two germ nuclei. Five sections are involved, and the male and female nuclei are indicated by the appropriate signs. The male component, distinguished by its accompanying aster, exhibits five nucleoli and the female thirteen. The volume of the female component is also much greater than that of the male component. I believe, therefore, that we have here an undeniable case of fusion of a partial sperm nucleus with an entire egg nucleus.

In the stage of the first cleavage spindle of eggs centrifuged just before penetration of the spermatozoön we have three classes of eggs, aside from a very few polyspermic eggs: (1) Some with a more or less normal cleavage spindle; (2) some with a monaster centered in a group of chromosomes; (3) some without any trace of astral radiations although chromosomes are formed. In a particular lot of eggs (7.4 of 1911) of which 10 per cent segmented, at the stage of the first cleavage the first class is rare, the second class is quite common, and the third is the most frequent condition. The first class evidently corresponds to the 10 per cent of eggs that segmented.

The two latter classes are illustrated in figs. 43 and 44. Fig. 43 is a camera drawing of the three sections of the nucleus of a single egg belonging to the third class. The eggs of the first class of the same lot were in various stages of the anaphase and telophase of the first cleavage. There appear to be fourteen chromosomes, the number usually found in the maturation spindles; the position near the animal pole proves it to be the egg nucleus. The absence of the sperm nucleus is readily demonstrated. There is an entire absence of all radiations in the cytoplasm. Both

polar bodies are present. This condition, as I have already said, is the commonest condition in such a lot of eggs, and both polar bodies are invariably present.

Attention may be directed to the fact that each chromosome is embedded in a homogeneous ground substance of about the same tint, in the iron haematoxylin stain employed, as the cytoplasm. Evidently, each chromosome with its surrounding matrix corresponds to a single chromosomal vesicle of the early egg nucleus; the numbers are the same. The chromosome of the succeeding cell generation arises within the substance of the chromosome of the preceding generation in this case.

The second class of cases, monasters, is illustrated in fig. 44. I was at first inclined to think that these might be due to fertilization with partial sperm nuclei, especially as the degree of development of the monaster shows a wide range of variation. But careful study of the eggs concerned showed that the first polar body had invariably failed to form, and the second was always present alone. About twenty-five cases of this kind have been examined without a single exception occurring.

In the second study of this series (Lillie, '11) I have described the cause of failure of the first polar body. This condition occurs in eggs centrifuged just before the formation of the first polar body. The first maturation division may then take place within the egg forming two nuclei, and the second maturation spindle which involves both nuclei is a tetraster (Lillie, '11, fig. 6). The second polar body is formed from one pole of the tetraster and three nuclei are left in the egg, which soon unite. Under these circumstances, if the sperm nucleus be absent, a more or less feeble monaster may develop at the time of the first cleavage; though in a few cases where only the second polar body was formed, no signs of radiations were found.

There is thus a more or less striking difference in the behavior of the egg nucleus in those cases where both polar bodies are formed and those in which the first polar body is suppressed. It is a very interesting problem whether the formation of the monaster in the latter case is a purely quantitative relation, due to the larger number of chromosomes present in such cases? There

is considerable variation in such cases in the size of the second polar body and the quantity of the chromatin which it contains, and corresponding differences in the number of chromosomes left in the egg. But the degree of development of the monaster is not a function of the number of chromosomes in the egg; and there are cases in which no aster formation is associated with a larger number of chromosomes in such eggs, and a well developed monaster with a smaller number. It is possible that there may be a qualitative relation depending on what chromosomes are extruded in the second polar body; but in view of the complicating considerations resulting from possible injurious effects of centrifuging itself, no definite conclusion on this point seems possible.

It is, in any event, certain that the sperm nucleus is absent in both the second and third classes of eggs.

As regards the effect of the partial sperm nuclei on the cleavage process we are therefore reduced to the class of cases in which a cleavage spindle is actually formed. In observing the living eggs I was struck with the fact that the cleavage of many centrifuged eggs tends to be irregular or partial, especially of those centrifuged at the time when injuries to the spermatozoön were to be expected. And in the sections I find many cases of partial cleavage. The cleavage of many also stops in the two-cell stage. It is natural to suppose that such partial cleavages are the result of fertilization with partial sperm nuclei, seeing that we know from the data recorded above that there is not even the least indication of cytoplasmic cleavage in the entire absence of the sperm nucleus. A rigorous demonstration of such a conclusion would, however, require a cytological analysis in which the number of chromosomes in the different cleavage spindles of normal and partially segmenting eggs should be compared; the relative sizes of the karyokinetic figure, and possibly other data, should also be taken into account. Unfortunately, the material preserved for this study is in too advanced a stage to make an exhaustive study of these relations, and this part of the investigation must therefore be postponed.

It must be admitted that other causes than fertilization with partial spermatozoa might be responsible for the partial cleavage,

e. g., injury of certain kinds caused by the centrifuging, possibly abnormal maturation, or a general systemic disturbance of the cytoplasm. But this does not seem very probable, since eggs centrifuged at times when injury to the spermatozoön is not to be anticipated do not exhibit the partial cleavage, at least to the same extent. I would therefore regard it as probable, though not proved, that partial sperm nuclei tend to produce more or less defective cleavage.

GENERAL DISCUSSION

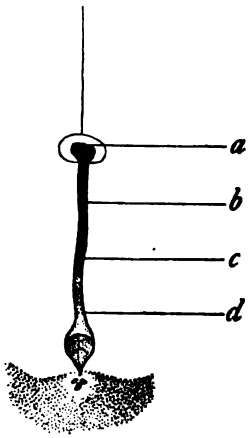
1. The centrosome theory of fertilization

The centrosome theory of fertilization is still accepted by most morphologists in spite of the doubts that have been thrown on the theory of the permanence and genetic continuity of the centrosome by the production of asters in unfertilized eggs (Morgan, Wilson, etc.), and by the studies in artificial parthenogenesis. The theory that the spermatozoön introduces an extra-nuclear centrosome destined to become the organ of cell division of the oösperm is confronted for the first time, in the results of the foregoing study, with a crucial experimental test. And the result that even small parts of the sperm head produce a typical centrosome and aster in the egg cytoplasm conclusively demonstrates the inadequacy of this conception of fertilization. So long as similar experiments on other forms are lacking, there is no reason to believe that the production of the sperm centrosome depends upon any different principle in Nereis than in other forms.

Meves ('11*a*) demonstrates that the sperm aster does not arise in connection with the so-called middle piece of the spermatozoön in the case of the sea-urchin, but at the base of the sperm nucleus itself, the middle piece being already separated from the nucleus and lying to one side at the time that the sperm aster arises. These results, while inconclusive in themse ves, are nevertheless distinctly unfavorable to the existence of an extra-nuclear centrosome as the cause of formation of a sperm aster in this classical case. Further, one can say without fear of successful contradiction that in no animal has it been shown that the

sperm aster arises around an extranuclear centrosome of spermatic origin. There is, therefore, no reason for assuming that the experiments on Nereis have revealed an exceptional condition.

Either, then, the centrosome theory of fertilization must be rejected in toto, or the sperm nucleus must be regarded as a centronucleus in Boveri's sense (Boveri '00), i. e., as including the centrosome. If we examine the latter point of view we see that it would be necessary to assume that centrosomes exist in the sperm nucleus at every level, for the sperm aster can form at any level as demonstrated by the experiments. The diagram (text fig. 4) will make this clear. It represents the sperm nucleus in the form that it possesses as penetration is nearly completed (cf. fig. 8); normally the sperm aster arises at point *a*, but if *ab* be removed then at point *b*, if *ac* be removed at *c*, if *ad* be removed at *d*, etc. But the assumption that centrosomes exist throughout the nucleus and condition this phenomena would seem to be merely an exaggerated morphological point of view with reference to a problem that is, after all, fundamentally physiological, viz.: by virtue of what properties does the sperm nucleus exercise this effect? If the great mass of experimental cytological data favored the view of the permanence of the centrosome, the con-



Text fig. 4

ception of the centronucleus might legitimately be extended to cover the present case, but this cannot be said to be true; and in the present state of our knowledge such an explanation would appear forced and merely formal.

The formation of the sperm aster takes place on the boundary between nucleus and cytoplasm, and as we have seen (fig. 41), there are very definite quantitative relations between the bulk of the partial sperm nuclei and the degree of development of the aster and the size of the centrosome. This relation leads to the conclusion that if intranuclear centrosomes are the causes of the formation of the sperm aster, not only must they exist at every level, but also that they must decrease in size from the base to the apex of the sperm nucleus! The formation of the sperm aster on the boundary between nucleus and cytoplasm and the quantitative relations existing between size of the nucleus and of the aster demonstrate, it seems to me, that the centrosome and aster owe their existence to an interaction between nucleus and cytoplasm, and not to any third element. All the observed relations in the case of Nereis harmonize with this point of view.

The production of astrospheres remote from the nucleus in the experiments of Morgan ('96 and '99) and others show, it is true, that the nucleus is not necessary for the production of such phenomena. These asters are apparently very temporary formations, and evidence that their central bodies may divide like the centrosome of the sperm aster, or other centrosomes associated with nuclei, is lacking. Nevertheless, it seems probable that fundamentally similar physiological causes are at the foundation of both sets of phenomena, and we can only assert our profound ignorance of what these causes really are.

2. The polarity of the sperm-nucleus

We may use the term polarity to describe the fact that the sperm aster arises invariably at the most basal point of the sperm nucleus, whether it be entire or partial. This phenomenon corresponds accurately to the general features of polarity of ova or lower organisms, as, for instance, the formation of oral organs at the oral end of a cut piece, etc.

It might, perhaps, seem possible at first thought that the aster appears in this position with reference to the partial sperm nuclei because we have here a broken surface; this is at least a condition in which this particular point of the surface of the partial nuclei differs from the remainder of its surface. But in the entire sperm nucleus, where there is no broken surface, the position is always the same. Moreover, some ten or fifteen minutes elapses after entrance of the sperm head before the aster becomes visible, and in this time the nucleus has changed form so as to produce a pointed extremity (figs. 33 *a*, 36, 37, 40, etc.) in the position where the aster is to appear whether the nucleus be entire or partial. This is perhaps sufficient evidence of repair of the wounded surface.

We have to seek some more profound cause for this localization, and I believe that it must be regarded as a special case of organic polarity to be explained like other cases on the basis either of gradation or orientation of materials. From this point of view the nucleus would possess an immanent structure determining the location of aster formation and therefore the plane of division of the nucleus.

3. Theory of fertilization

I have pointed out repeatedly in these studies that fertilization involves two phases, viz.: an external phase prior to entrance of the spermatozoön, in which certain cortical changes are produced in the egg, and an internal phase, following penetration, involving a complex series of phenomena. As I pointed out in the introduction, this paper is a contribution to the analysis of the second phase. On the basis of experiments on artificial parthenogenesis and hybrid fertilization, Loeb has made a similar distinction of two phases, and so far the results of what we may call the biological and the physico-chemical analyses of fertilization are in accord.

If we reject the centrosome theory of fertilization, as I believe we are compelled to do, what point of view from the side of the biological analysis shall we put in its place? The theory of the

internal phase of fertilization must proceed from the fundamental fact of the difference in behavior of the sperm nucleus and the egg nucleus in the cytoplasm of the egg. The former induces aster formation and karyokinesis; the latter does not. Let us recall the facts in the case of Nereis briefly again: (1) Even minute fragments of the sperm nucleus cause the formation of an aster with a centrosome capable of division. (2) If the sperm nucleus be prevented from entering, the egg nucleus may indeed form chromosomes but no aster arises, provided that both polar bodies are formed.[2] (3) We may add from experiments on other forms that in the absence of the egg nucleus the sperm nucleus behaves the same as in its presence.

Clearly, then, there is some difference, associated with their sex-origin, between these two nuclei; and the most direct form of interpretation of this difference is that which identifies it with the fundamental sex characters which inhere in every cell. In other words, the sperm nucleus has the character maleness, whatever that may be, and neither the egg nucleus nor cytoplasm possesses this character. It makes no difference that half the spermatozoa may carry the *factor* for femaleness and half for maleness. The distinction between *character* and *factor* is clear. It may be, on the other hand, that ova and spermatozoa acquire in the course of gametogenesis special differentiating properties that are the cause of the fertilizing power of the spermatozoön.

However we may conceive the demonstrated difference between the sperm nucleus and the egg nucleus, it is obvious that there is a lack of interchange between the egg nucleus and the egg cytoplasm that conditions the inhibition of the unfertilized egg. In some way, then, the maturation divisions of the egg must have removed certain reacting constituents of the germinal vesicle, or have brought about certain cytoplasmic changes in the egg, because we have perfect karyokinetic phenomena in the maturation divisions and a sudden cessation thereafter.

[2] The monaster that arises after suppression of the first polar body with prevention of entrance of the sperm nucleus forms a special problem which we need not consider here.

But it is clear at least that the maturation of the egg does not differ from the maturation of the spermatozoön in this respect. In both cases capacity for further cell-division is lost after the second maturation division, and it is quite natural, certainly, to postulate similar causes for this phenomenon in both sexes. If, as the results of the present study indicate, karyokinesis is the result of a certain *qualitative* nucleo-plasmic relation (to be distinguished from R. Hertwig's *quantitative* nucleo-plasmic relation) we have to postulate in both cases a disturbance of this relation. And as this relation must be conceived as a chemical interaction of some kind, precipitated possibly by rhythmical changes of permeability of the nuclear membrane, the alteration in question must involve either the establishment of an impermeable condition of the nuclear membrane or a chemical change in nucleus or cytoplasm. But we have seen in Nereis, that, even when the membrane of the nucleus of the mature egg breaks down, no karyokinetic phenomena follow, unless the egg is fertilized. So the phenomenon of cessation of division can hardly be conceived as conditioned by the membrane alone.

The egg-cell and the spermatid are not the only cells that lose the capacity for division in the course of development. In the course of senescence all cells lose this capacity, and studies in cell-lineage have shown that certain cells entirely lose the capacity for division in very early stages. I need cite only the case of the so-called turret cells in Crepidula (Conklin '97), which are formed in the sixteen cell stage, and which divide only twice during the cleavage period. Mead has called attention to similar cases in his studies of cell-lineage in Annelids (Mead '98). Cessation of division cannot be a problem of centrosome or no centrosome in such cases; nor yet in the case of the spermatid. A much more profound physiological cause must be involved.

Constructive metabolism has come essentially to a standstill in the mature gametes; the rate of metabolism in the mature unfertilized egg as tested by oxygen consumption is many times less than that of the fertilized egg (Warburg, '05). Child ('11) cites, as conditions that lower the rate of metabolism, decrease in permeability, increase in density, accumulation of relatively inactive

substances, etc., but we know that constructive metabolism is also impossible in the absence of a nucleus, and we may conclude from many facts, as Conklin ('12) expresses it, that "rapid and intimate interchange between the chromatin and the protoplasm is the condition of rapid metabolism and ex hypothese of rejuvenescence; slow interchange is the condition of slow metabolism, and of senescence." It is on account of the slowness of such interchange between nucleus and cytoplasm, as I believe, that the unfertilized egg is inhibited from development. The internal function of the spermatozoön in development is to restore the condition of active and intimate interchange between nucleus and cytoplasm. Aster formation and karyokinesis are evidences of such restoration. The sperm nucleus and egg cytoplasm are immediately capable on union of such interchange, and as the fertilization process proceeds the egg nucleus is drawn in.

We are led, then, to the following point of view with reference to the internal phenomena of fertilization, viz.: in both the sperm and the egg cell as the result of maturation the capacity for the nucleo-plasmic interaction necessary for construction metabolism has been lost. But such interaction takes place between the sperm nucleus and egg cytoplasm, and this initiates the internal phenomena of fertilization. The egg nucleus also is drawn into the karyokinetic phenomena in one of two ways, either that the sperm nucleus has so altered the egg-cytoplasm that karyokinetic reaction between the egg-nucleus and its own cytoplasm can now follow, or that copulation of the germ nuclei results in a change in the egg nucleus that restores its capacity for the necessary nucleoplasmic reaction.

In his experiments on constricting fertilized eggs of the sea-urchin between the germ nuclei, so that the copulation of the latter was prevented, Ziegler ('98) has shown that the egg nucleus becomes surrounded by cytoplasmic radiations which rhythmically appear and disappear synchronously with disappearance and reappearance of the nuclear membrane. These observations indicate a change produced by the sperm nucleus throughout the egg cytoplasm, inducing partially but not completely the rhythmical series of successive karyokinetic divisions. Other obser-

vations too numerous to mention demonstrate a very profound effect of the sperm nucleus on the egg cytoplasm, perhaps none more strikingly than Herlant's ('11) recent observations on the control of definite cytoplasmic areas (spermatic energids) by spermatozoa in di- and tri-spermic eggs of the frog.

Ziegler's observations then indicate that the egg nucleus reacts to the egg cytoplasm when altered by the spermatozoön, but *incompletely*. It seems probable, therefore, that copulation of the germ nuclei also involves an interaction between them that completes the fertilization phenomena. It is interesting to note that, though the chromosomes form in Nereis from the egg nucleus after the spermatozoön has been removed, they are not set free in the cytoplasm as they are after copulation with the sperm nucleus, but each is embedded in a matrix, and thus presents quite a different appearance from the normal. We may perhaps find in this fact, indicating lack of reaction between the chromatin and the cytoplasm, some evidence that the completion of fertilization involves interaction between the germ nuclei also.

It remains to inquire briefly how this analysis compares with the analysis of fertilization given by experiments in artificial parthenogenesis? In the first place we may note again that there is perfect agreement in the general fundamental distinction of two phases in the fertilization process as made first by Loeb, viz.: the cortical change which may be induced before penetration, and the internal changes, which follow penetration. As regards the cortical change, the view of Loeb ('09) that it is essentially a cytolytic change appears to me less fundamental than the view of R. S. Lillie ('11), that it is essentially an increase of permeability. One can readily understand that cytolysis should follow very rapidly on an increase of permeability induced by chemical means, which may be much greater than that normally induced by the spermatozoön, if such increase be not secondarily regulated. And in any event, if interchange between the egg and its medium be set up by increase of permeability, in a condition of inactivity of the nucleus, such as exists in the unfertilized egg, the resulting metabolism must be of a destructive character and so lead to a relatively rapid death of the egg as compared with eggs in which

the cortical changes have not been induced. The conception of Bataillon ('10), moreover, that the egg excretes certain inhibiting substances contained in its cortex, as a result of the cortical change, is quite readily included in this point of view; indeed, the jelly excreted by the egg of Nereis as a result of the external stimulus of the spermatozoön would obviously hinder free interchange between the egg and the medium so long as it exists within the egg as a thick cortical layer.

The second phase in fertilization has been treated by Loeb and R. S. Lillie. Loeb's interpretation is that the second agent in artificial parthenogenesis serves to check the tendency to cytolysis set up by the first agent; and he extends this point of view to the two phases of normal fertilization. In this opinion he is followed by Godlewski ('11), who has shown that the cytolysis, which follows on fertilization of sea-urchin eggs with sperm of Chaetopterus, can be checked, and parthenogenetic development induced, by a brief treatment of such cross fertilized eggs with hypertonic sea-water. R. S. Lillie ('11) regards "the critical change in the egg, to which membrane formation and the initiation of cleavage are due, as a well marked and rapid increase in the permeability of the plasma-membrane." This tends "to destroy the normal osmotic equilibrium and allow abnormal diffusion of substances into and out of cells" leading to derangement and eventual destruction of the chemical organization of the latter. And he regards it as an unavoidable conclusion that one essential effect of the after treatment with hypertonic sea-water is to restore the normal permeability.

There is considerable similarity in these points of view; both regard the second agent in parthenogenesis essentially as a regulatory factor. Godlewski's very striking results on the combination of cross fertilization and artificial parthenogenesis lead to the same kind of conclusion; to quote this author ('11):

Wir haben gesehen, dass weder die Kreuzbefruchtung allein, noch die so kurz dauernde Exposition in hypertonischer Lösung allein ausreicht, um die Entwicklung auszulösen. Erst durch die Kombination der beiden Faktoren ist der ausreichende Anstoss zur Entwicklung gegeben. Ich sehe in diesen Tatsachen die Bestätigung der von J. Loeb aufgestellten Hypothese, dass der Process der Entwicklungserregung sich in zwei

Momente zerlegen lässt—im ersten findet die Cytolyse, im zweiten die Regulierung der im ersten Akt bereits eingetretenen inneren Reaktionen im Eiorganismus statt.

There is nothing in the results of these authors inconsistent with the idea that the regulating effect of the second agent in artificial parthenogenesis may be attained through the re-establishment of normal interchange between the nucleus and the cytoplasm, which is the view to which I have been led by the results described in this series of papers. And this suggestion contains the possibility of a complete accord between the results of the analysis of fertilization by methods of artificial parthenogenesis, and the more direct method of analysis contained in my papers.

If the egg be put in a healthy metabolic state by the resumption of normal nucleo-plasmic relations following the penetration of the spermatozoön, it can no doubt regulate its own external affairs. And so it seems to me that the regulation of cortical permeability and cytolysis is probably a secondary effect of the re-establishment of normal nucleo-plasmic interchange. Even in artificial parthenogenesis, where the sperm nucleus is lacking, the action of the second agent may proceed in the same way, by causing the re-establishment of normal interchange between the egg nucleus and cytoplasm, restoring thus healthy conditions, which then regulate the cortical changes. But whether the tendency to cytolysis be checked thus secondarily, or by some direct effect of the second agent on the cytoplasm, development certainly cannot proceed without the establishment of normal metabolic interchange between the nucleus and the cytoplasm, and this must certainly be regarded as a fundamental function of the second agent in artificial parthenogenesis.

To sum up the conclusions in a sentence we may say: the action of the spermatozoön in fertilization involves two distinct phases, the first of which may be effected before penetration and brings about a sudden and marked increase in permeability of the egg-membrane; the second, which follows after penetration, consists essentially in the establishment of normal interchange between nucleus and cytoplasm, and consequent normal regulation of all the activities of the cell.

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DESCRIPTION OF PLATES

All figures were drawn with the camera at stage level with Zeiss Apochromat 1.5 mm. oil immersion objective, and No. 12 compensating ocular, except where otherwise stated. All figures, except 1, from sections of inseminated eggs of Nereis limbata. All sections from eggs killed in Meves' fluid and stained in iron haematoxylin. Plates 1 to 5 illustrate the third study; 6 to 11 the fourth study.

PLATE 1

EXPLANATION OF FIGURES

1 Spermatozoa of Nereis from preparations fixed in Gilson's fluid and stained in safranin and Säure-violet. 1 *a*, the entire spermatozoön; 1 *b* and 1 *c* show the excentric attachment of the tail to the middle piece; 1 *d*, basal view of the sperm-head showing ring-shaped middle piece and attachment of tail. *h. c.*, head-cap; *m.p.*, middle-piece; *p*, perforatorium. It may be noted here that the form of the perforatorium in the living spermatozoön is more like the spike of a helmet.

2 *a*, 2 *b*, 2 *c* From three eggs fifteen minutes after insemination. The entrance cone is well developed and stains dark, homogeneous. The perforatorium has pierced the membrane, at least in 2 *a* and 2 *c*, but is not embedded in the cone. Note variations in appearance of the middle-piece. Tail not seen.

3 *a*, 3 *b* From two eggs twenty-seven minutes after insemination. The cone is retracted, but stains as before. The perforatorium stains more strongly than in fig. 2, but it has not yet entered the substance of the cone. The tails were very distinctly seen in these preparations.

4 Thirty-four minutes after insemination. The perforatorium has now entered the substance of the cone, and granules are beginning to appear in a smaller lighter area of the cone surrounding its tip.

5 *a*, 5 *b* From two eggs thirty-seven minutes after insemination. The clear space in the cone is now larger and the granules at the tip of the perforatorium more numerous; 5 *a*, from an almost tangential section, so that the vitelline membrane was obscured. Note the double perforatorium; 5 *b*, middle piece shows no granules; cf. 2 *b*.

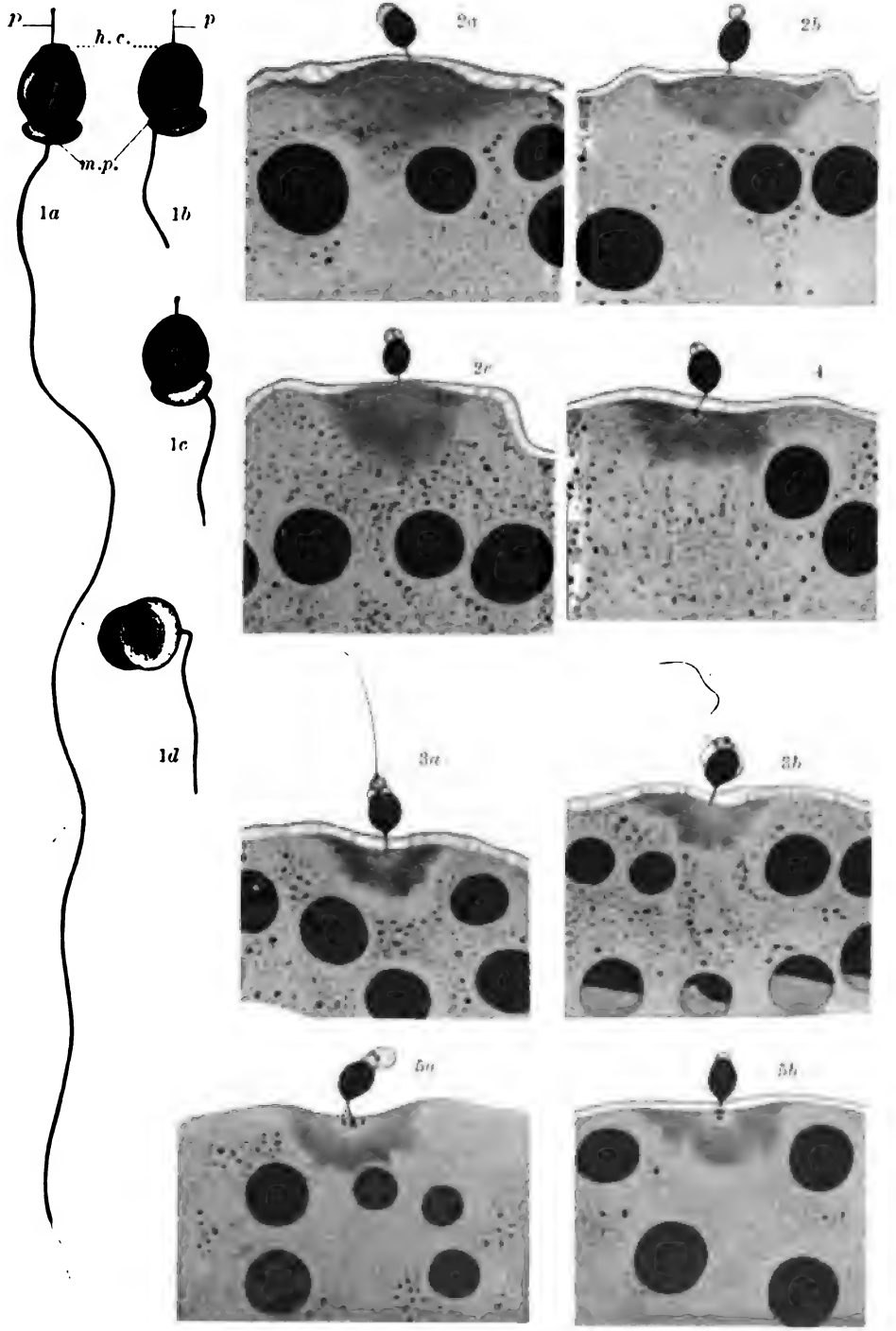
p
h. c.
p
m.p.
1a
1b
1c
1d
2a
2b
2c
4
3a
3b
5a
5b

PLATE 2

EXPLANATION OF FIGURES

5 *c* and 5 *d* From two eggs thirty-seven minutes after insemination; cf. 5 *a* and 5 *b*.

6 Forty-eight and one-half minutes after insemination. The entrance cone has begun to sink into the egg, drawing the head of the spermatozoön after it.

7 Forty-eight and one-half minutes after insemination. Later stage of penetration of the spermatozoön.

8 Forty-eight and one-half minutes after insemination. Still later stage of penetration.

9 *a* and 9 *b*. Two drawings of the same section; 9 *a* drawn with Zeiss 2 mm. apochromatic objective and no. 4 compensating ocular; 9 *b*, the part of 9 *a* containing the spermatozoön, drawn with Zeiss 1.5 mm. apochromatic objective and no. 12 compensating ocular. Fifty-four minutes after insemination. The sperm head has completed its penetration, and its base is some distance from the periphery; but the middle-piece remains outside. Anaphase of the first maturation division.

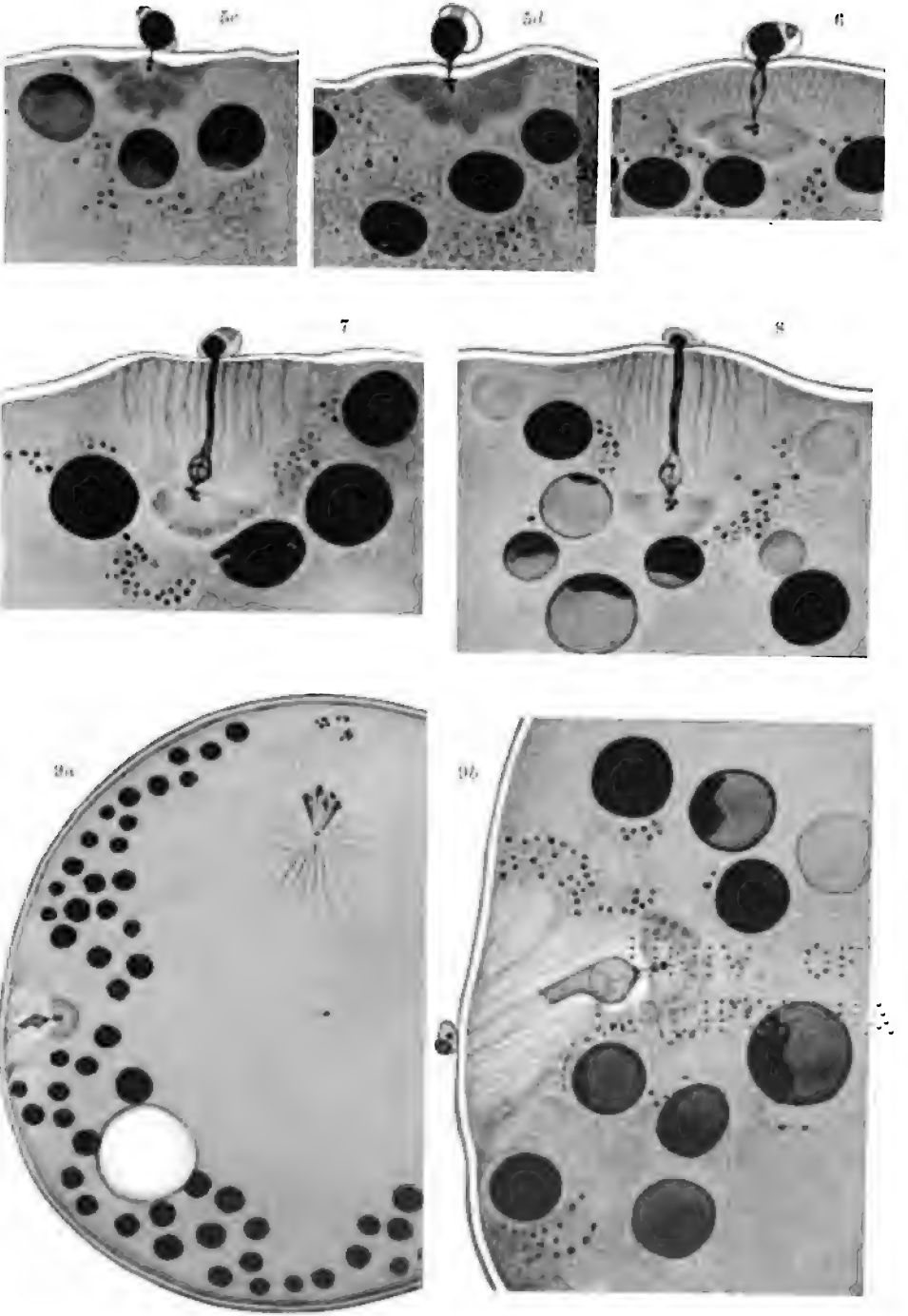

PLATE 3

EXPLANATION OF FIGURES

10 *a*, 10 *b*, 10 *c*, 10 *d* Four drawings from the same section; 10 *a* drawn with Zeiss 2 mm. apochromatic objective and No. 6 compensating ocular; 10 *b*, 10 *c*, 10 *d* drawn with Zeiss 2 mm. apochromat and no. 12 compensating ocular; 10 *b* is the sperm mucleus of the same egg; 10 *c* and 10 *d* are from the following sections to show the entrance point with tail and middle piece on the membrane. Fifty-four minutes after insemination. Rotation of the sperm head and cone, and origin of the sperm aster from the pole of the nucleus opposite to the cone. Prophase of the second maturation division.

11 Sixty-four minutes after insemination. The sperm head has penetrated within the layer of yolk, and has separated from the cone, which does not appear after this stage. In the next section another sperm centrosome and aster appear; the sperm centrosome has divided.

12 Sixty-seven minutes after insemination. Sperm amphiaster with equal poles; spindle formation beginning between egg and sperm centrosomes. Anaphase of second maturation spindle. Drawn at stage level with Zeiss 2 mm. apochromatic objective and compensating ocular no. 6.

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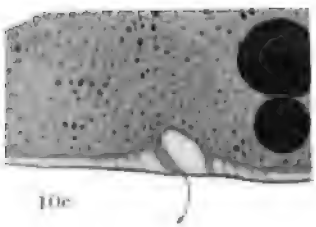

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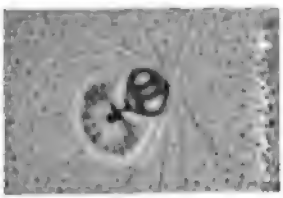

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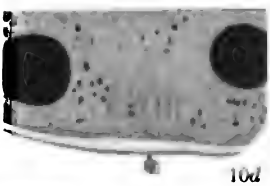

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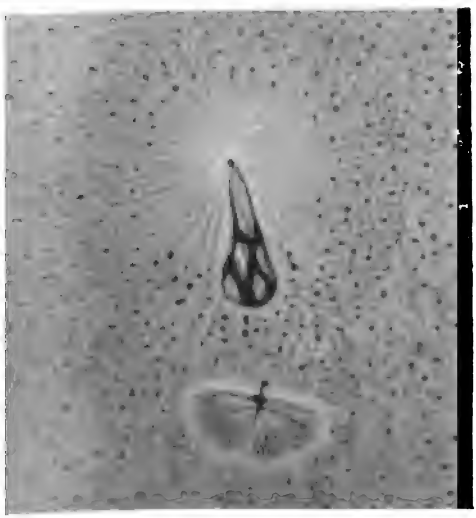

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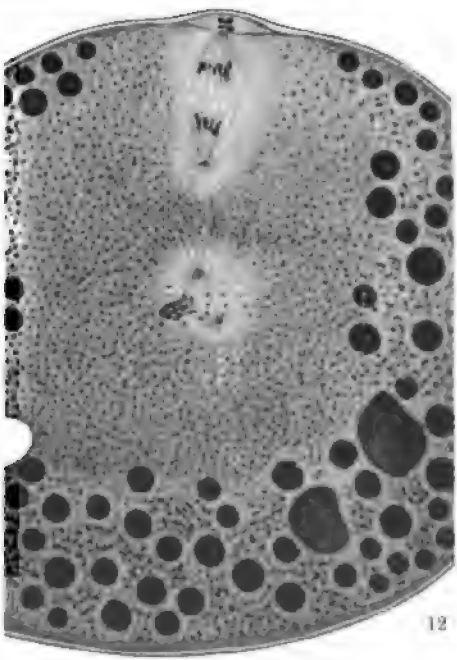

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PLATE 4

EXPLANATION OF FIGURES

13 Sixty-seven minutes after insemination. Later stage of sperm amphiaster with unequal centrosomes and asters. Double spindle formation between egg center and sperm centers. Drawn at stage level with Zeiss 2 mm. apochromat objective and compensating ocular no. 6.

14 *a* and 14 *b* Seventy-seven minutes after insemination. Two successive sections of the same egg. The second polar body is just formed. The egg aster is beginning to degenerate. The sperm asters are also less developed than previously, especially in the case of the smaller one. Sperm amphiaster in 14 *a*; sperm nucleus in 14 *b*. Drawn at stage level with Zeiss 2 mm. apochromat objective and compensating ocular no. 6.

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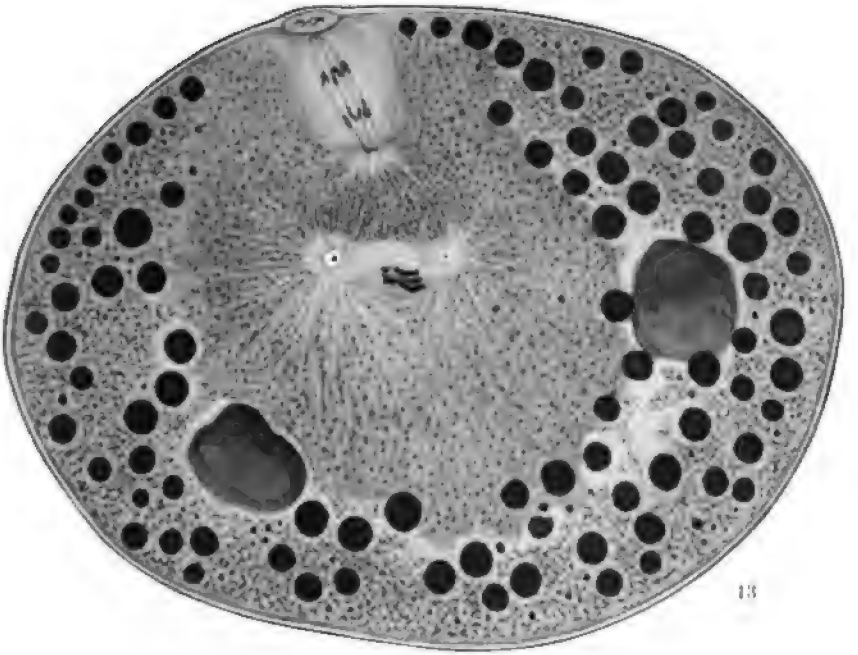

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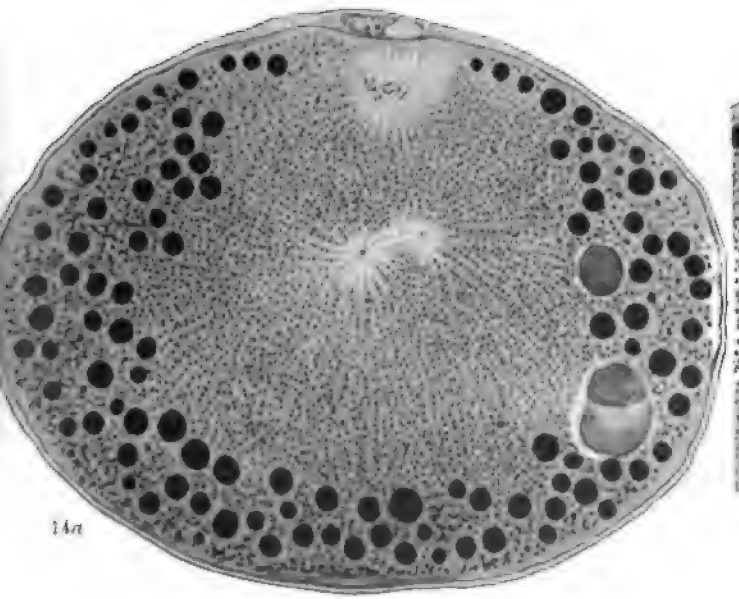

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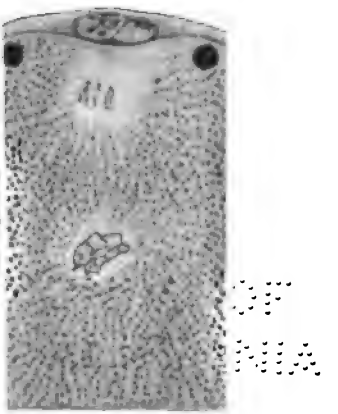

14b

PLATE 5

EXPLANATION OF FIGURES

15 Seventy-seven minutes after insemination. The two nuclei to the right above are parts of the egg nucleus not yet fused together. The sperm nucleus to the left below. The aster is the larger sperm aster; the smaller one could not be found. Drawn at stage level with Zeiss 2 mm. apochromat objective and compensating ocular no. 6.

16 *a* and 16 *b* Two successive sections of the germ nuclei, seventy-seven minutes after insemination. There is but a single aster derived from the larger sperm aster. Note the black granules in the neighborhood of the germ nuclei and aster. Drawn at stage level with Zeiss 2 mm. apochromat objective and compensating ocular no. 6.

17 Origin of the cleavage centers. The partition between the germ nuclei has disappeared. The larger aster is derived from the larger sperm aster. Drawn at stage level with Zeiss 2 mm. apochromat objective and compensating ocular no. 6.

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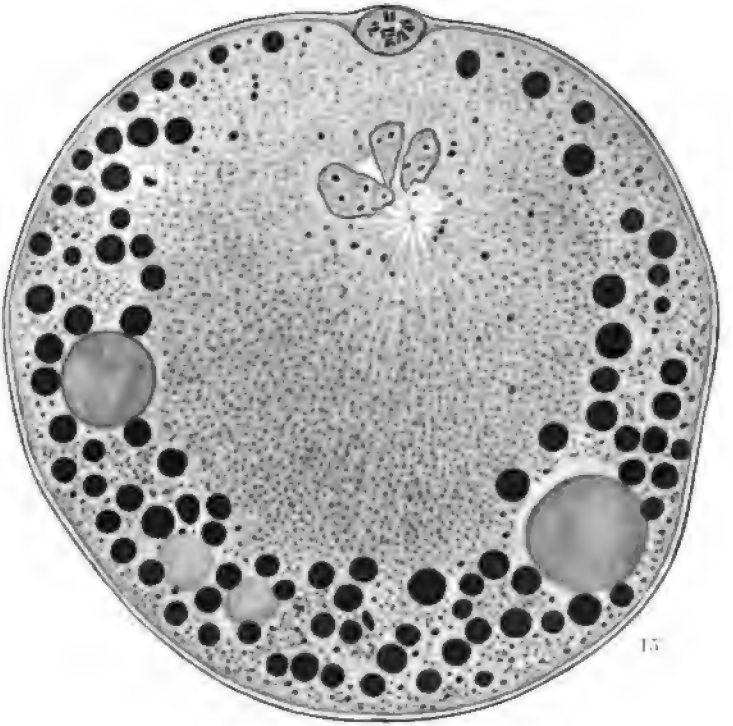

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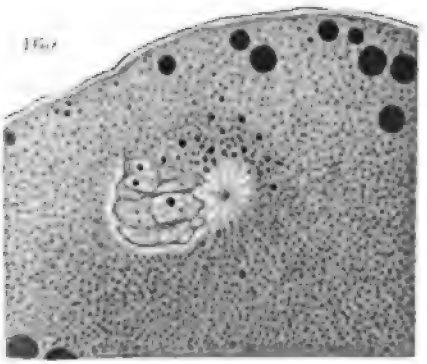

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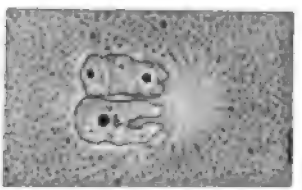

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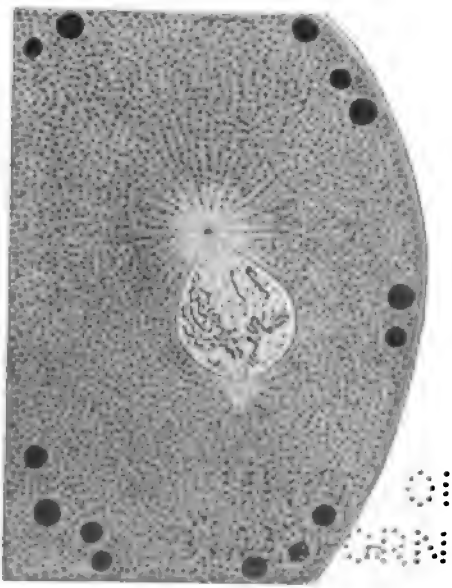

PLATE 6

EXPLANATION OF FIGURES

18 to 25 Show the effects of removal of the jelly by centrifuging on the spermatozoön attached to the egg. It will be noticed that the cone in this and the following plates, though just as well defined as the preceding, stains differently. The behavior of the cone is the same, however.

18 Entire spermatozoön present, drawn out to band. History: centrifuged 7200 revolution in thirty-five seconds, fifty minutes after insemination; preserved immediately.

19 The middle-piece and part of the base of the spermatozoön have been removed by the jelly. The protoplasm surrounding the cone has been raised in a protuberance, which happens not infrequently (cf. fig. 22). History same as fig. 18.

20 to 23 These figures show removal of increasingly large portions of the sperm head by the jelly. Each drawing from a single section of a separate egg. History same as fig. 18.

24 to 25 To show effects of removal of jelly by centrifuging after penetration has begun. History same as fig. 18.

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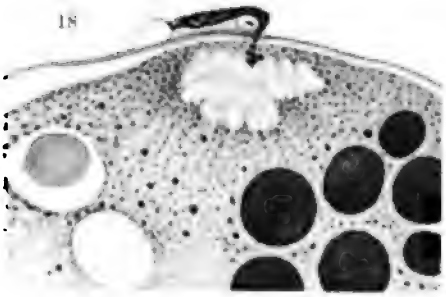
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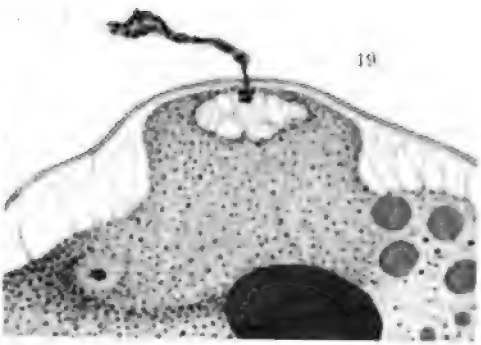
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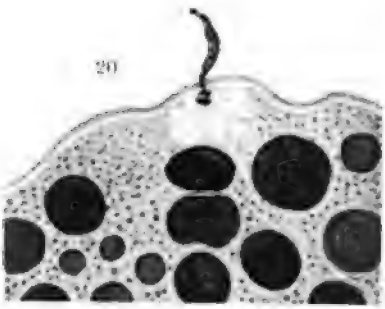
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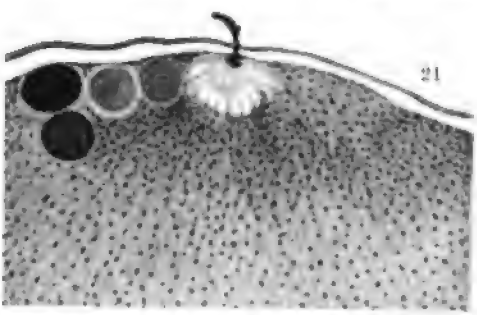
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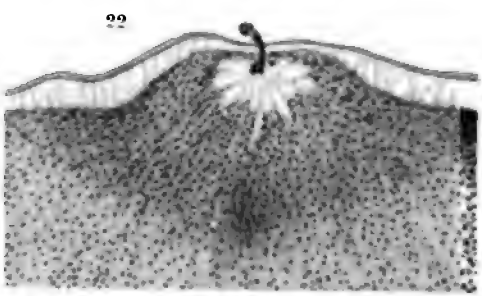
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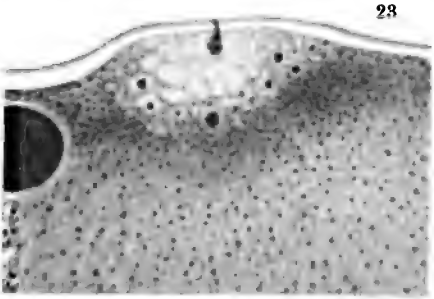
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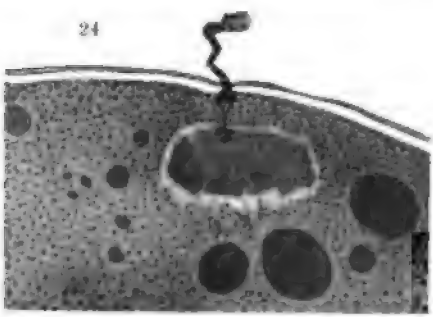
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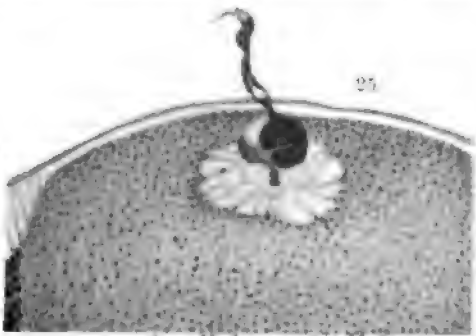
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PLATE 7

EXPLANATION OF FIGURES

26 Removal of external part of the spermatozoön by centrifuging in an advanced stage of penetration (cf. fig. 7). History: centrifuged 7200 revolutions in forty seconds, fifty minutes after insemination. Preserved fifteen minutes later.

27 to 31 To show early penetration of injured or partial spermatozoa. History: centrifuged 7200 revolutions in forty seconds, fifty minutes after insemination. Preserved fifteen minutes later.

27 Part of the spermatozoön has entered. The remainder is shown external to the membrane. The internal part is definitely divided in two. Rotation is beginning.

28 The part of the spermatozoön external to the membrane is nearly separated from the internal part, which is itself definitely divided in two. All parts a little swollen as shown by the tone of the stain. The rotation of the cone is beginning.

29 The internal part is apparently breaking off from the much larger external part of the spermatozoön. Cone in process of rotation.

30 A case in which only a small part of the spermatozoön has entered; the rest of the spermatozoön is lost. It represents a later stage of an injury similar to that shown in figs. 20 or 21.

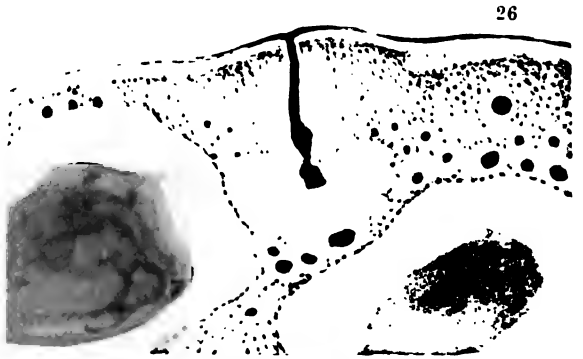
31 A case similar to fig. 30.

32 A somewhat later state of rotation of the cone than shown in preceding figures. History the same.

33 *a* and 33*b* Two successive sections of the same egg; the parts of the spermatozoön shown in the two sections are entirely separate. The proximal larger part (33 *a*) is proceeding with its rotation and development, leaving the base of the sperm head and the middle piece behind. History same as figs. 27–31.

34 Penetration stage of a sperm remnant preserved fifteen minutes after centrifuging.

40 Two partial sperm nuclei with asters associated with a single cone. Probably a later stage of a condition like that shown in figs. 27 or 28.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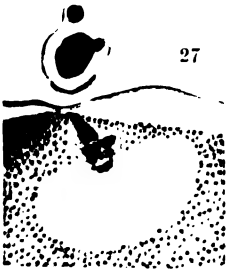
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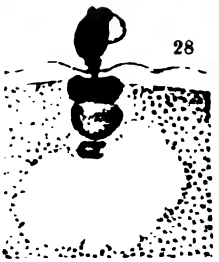
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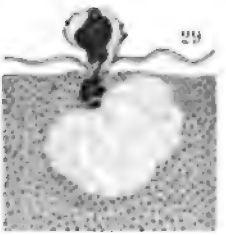
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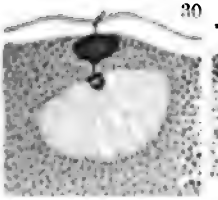
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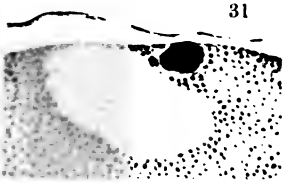
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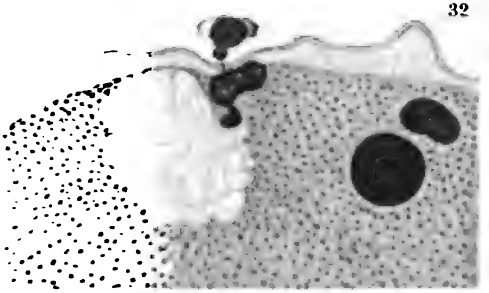
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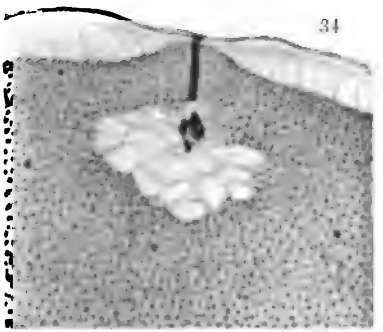
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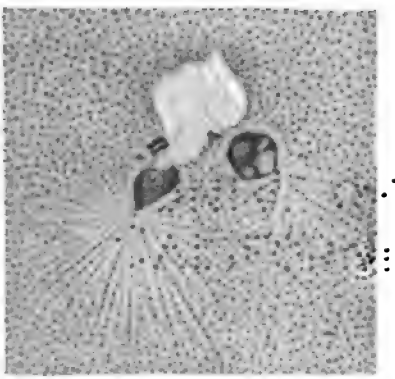
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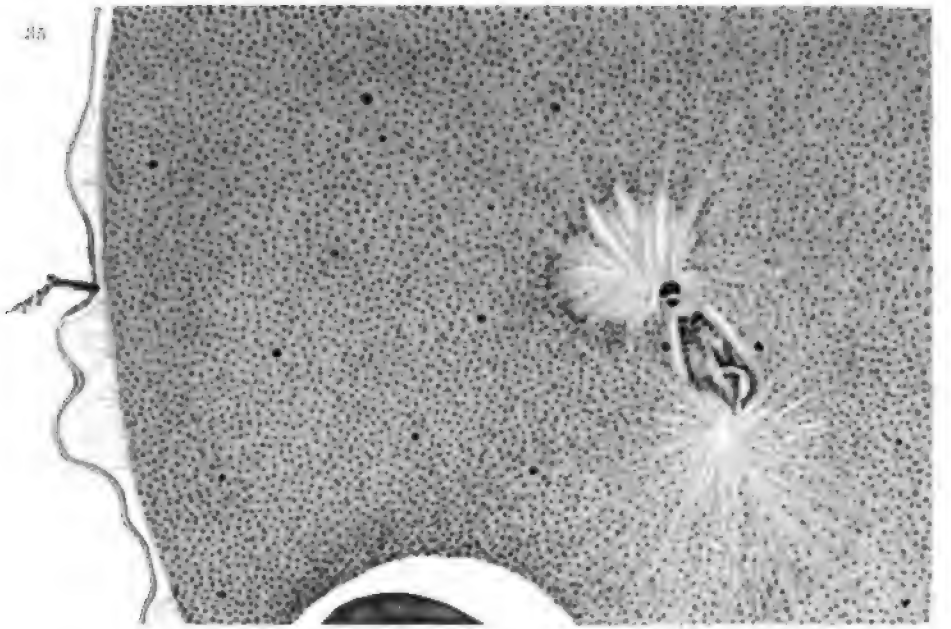
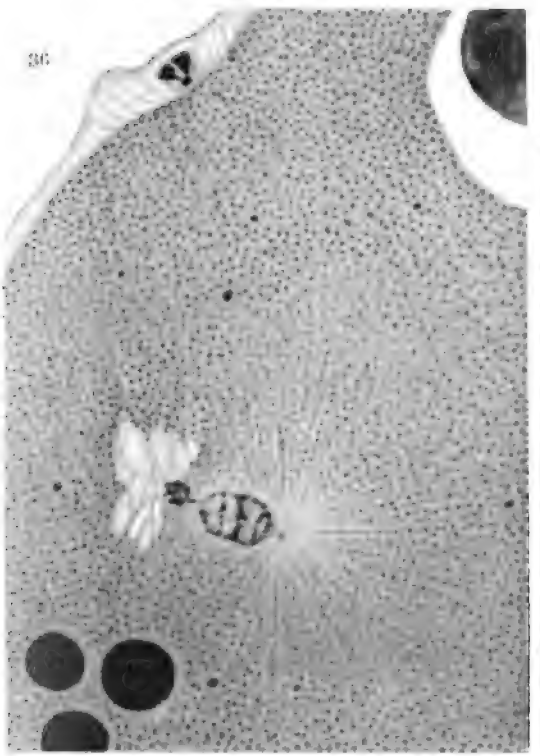
PLATE 8

EXPLANATION OF FIGURES

35 to 37 To show origin of asters in connection with partial sperm nuclei. In each case a remnant of the spermatozoön on the surface guarantees the partial nature of these sperm nuclei; note the variation in size. History: Centrifuged 7200 revolutions in forty seconds, fifty minutes after insemination; preserved fifteen minutes later. Fig. 36 is a combination of three sections.





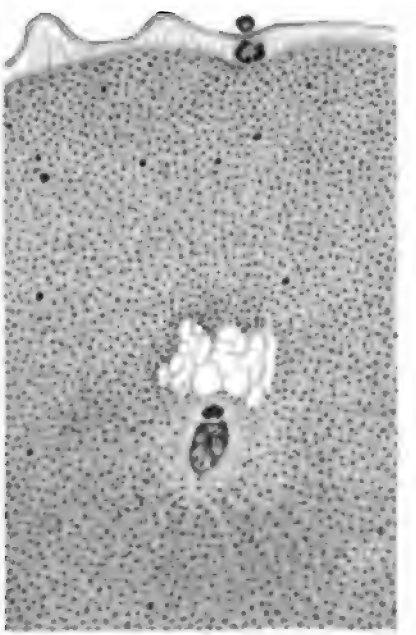


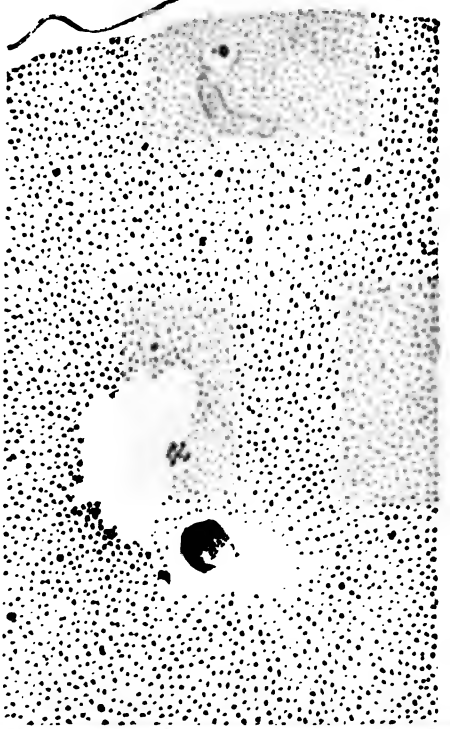
PLATE 9

EXPLANATION OF FIGURES

38 and 39 Two unusually small sperm nuclei with their asters, separating from the cones. From two eggs; compare the size of the entire sperm nucleus at this stage (fig. 11). History same as figs. 35 to 37.

41 Two sperm nuclei of unequal size from the same egg. Compare size of centrosomes and asters. History: Centrifuged 7200 revolutions in thirty-six seconds, forty-four minutes after insemination; preserved ninety-two minutes after insemination. Reconstruction of three sections.

38



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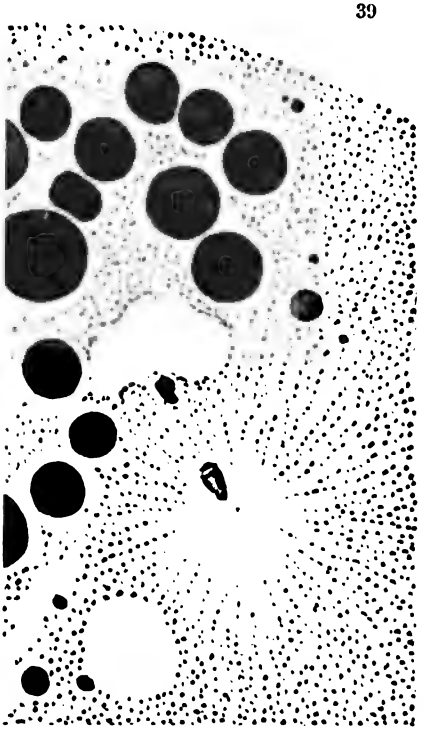

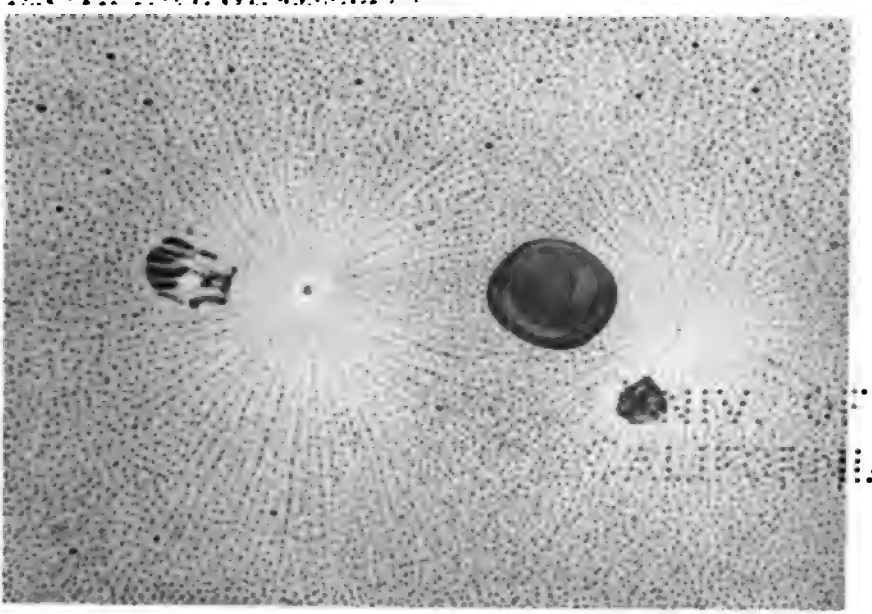

41

PLATE 10

EXPLANATION OF FIGURES

42 Five successive sections showing the entire germ nuclei of one egg. The male nucleus has five nucleoli, the female has thirteen. The line of apposition of the germ nuclei is seen in the third section. History: Centrifuged 7200 revolutions in thirty-six seconds, forty-four minutes after insemination. Preserved sixty-four minutes later.

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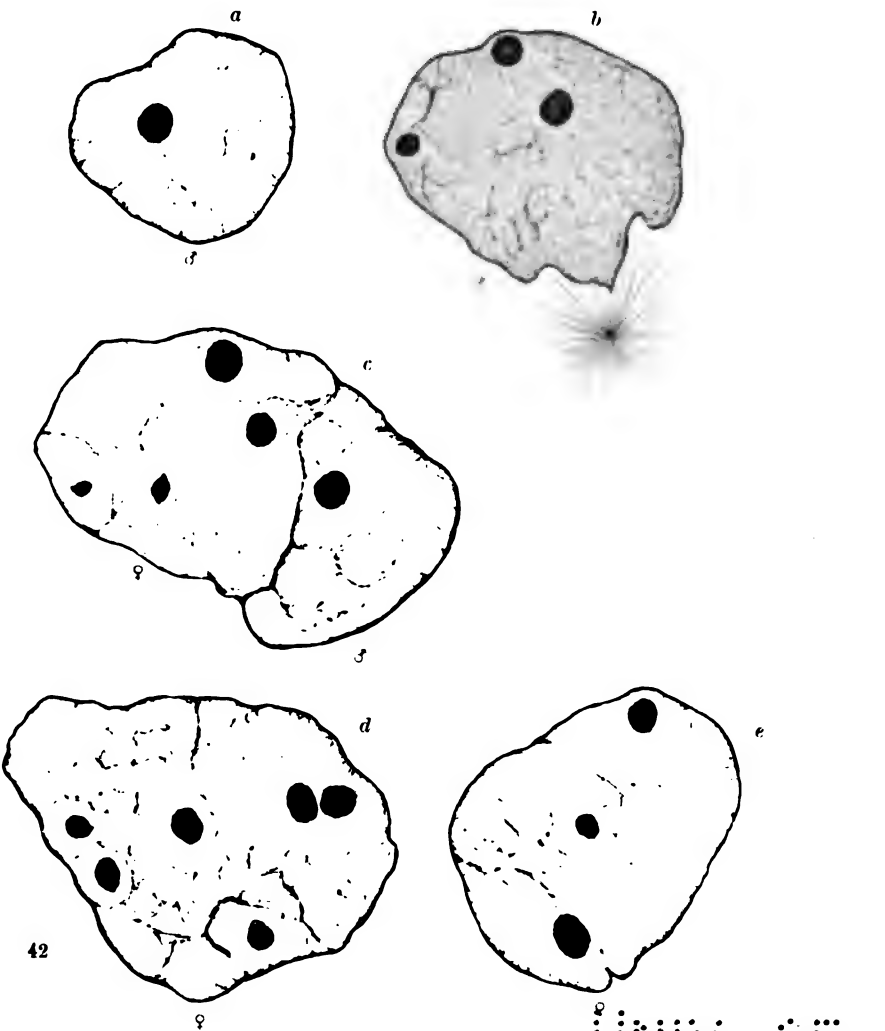

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PLATE 11

EXPLANATION OF FIGURES

43 Three successive sections showing the entire egg nucleus of an egg from which the spermatozoön was entirely removed by centrifuging. Both polar bodies formed. Fourteen chromosomes indicated. History: Centrifuged 7200 revolutions in thirty-seven seconds, forty-two minutes after insemination. Preserved sixty-five minutes later.

44 Three successive sections showing the entire egg nucleus of an egg from which the spermatozoön was entirely removed by centrifuging. The first polar body was not formed in this case, and a monaster arises around the egg-chromosome group. History same as fig. 43.

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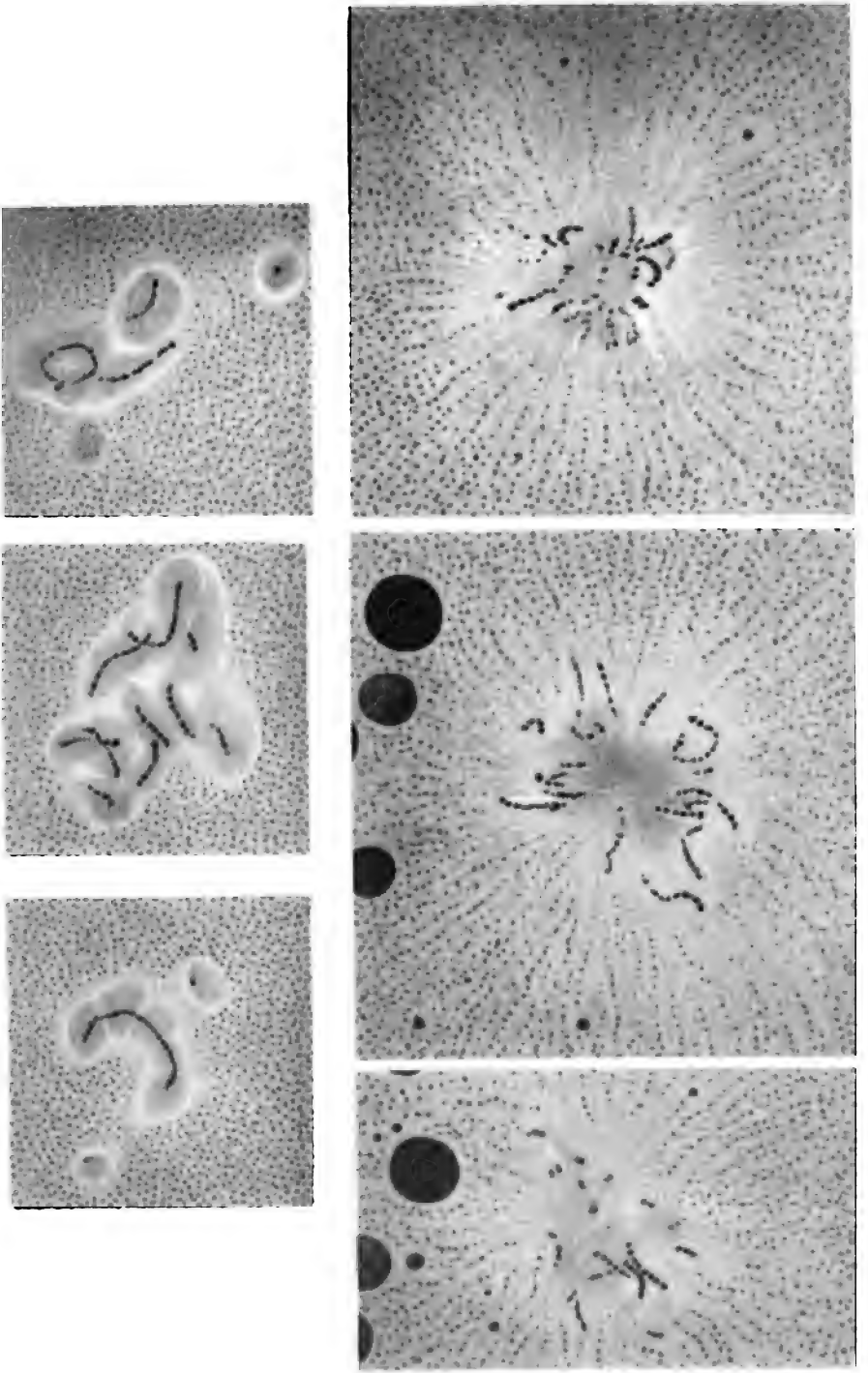

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STUDIES OF FERTILIZATION[1]

V THE BEHAVIOR OF THE SPERMATOZOA OF NEREIS AND ARBACIA WITH SPECIAL REFERENCE TO EGG-EXTRACTIVES

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[1] The earlier studies of this series bore the general title "Studies of fertilization in Nereis."

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I. INTRODUCTION

The earlier studies of this series dealt exclusively with Nereis and concerned the cortical changes of the egg, partial fertilization, the morphology of the normal fertilization and the fertilizing power of portions of the spermatozoon. They yielded certain positive results which I need not review, but they had convinced me that other methods than the ones usually in vogue, including the methods of artificial parthenogenesis, are needed for a closer approach to some fundamental problems of fertilization. Some incidental observations drew my attention to the study of the behavior of the spermatozoa, and investigation of the subject soon showed that the reactions of these minute active reproductive elements might furnish evidence of considerable significance. This study was begun in the summer of 1911 and continued throughout the summer of 1912 at the Marine Biological Laboratory.

With the publication of Loeb's first study on artificial parthenogenesis the study of fertilization entered upon a new phase which has not yet run its entire course. The tendency during this phase of investigation has been to regard initiation of development as the fundamental problem of fertilization; and the aim has been to discover the way in which the spermatozoon induces development of the egg. Hence the term 'chemical fertilization'

has come to be used loosely as practically synonymous with artificial parthenogenesis, as though a salt solution could take the place, and play the rôle, of the spermatozoon. This it can do obviously only with reference to the initiation of development, which, so far from being the only function of fertilization, is more properly to be regarded as a secondary function, or better a separate phenomenon which is sometimes associated with fertilization, sometimes not. On the one hand we may have initiation of development without fertilization, as in parthenogenesis and all asexual modes of reproduction, and on the other hand the phenomenon of fertilization without initiation of development is extremely common, as in the so-called winter eggs of Cladocera, Aphids and Rotifera, where fertilization is followed by a long resting period; the Protozoa and unicellular algae also offer many instances in which fertilization is the immediate prelude to a long resting stage.

The study of initiation of development by chemical means has yielded results of prime importance, and the consequent absorption in these problems has been an inhibiting factor in the analysis of other problems of fertilization. Thus, as spermatozoa are not necessary for "chemical fertilization,' the study of their behavior has been largely neglected. The problem of specificity has as a consequence been left almost entirely out of account, for there is no specificity in salts, or even in the blood sera of animals of other phyla; nevertheless specificity in reaction of sexual products is a much more nearly universal phenomenon of fertilization than initiation of development, and it is quite possible that the solution of this problem may furnish a valuable clue in the study of the latter problem. In any event, the time seems ripe for the development of new methods of attack on the fundamental problems of fertilization. The present contribution is a step in this direction. I have taken up the study of the behavior of the spermatozoa, because it represents, after all, considered in a broad sense, one-half of the problems of fertilization, and it seems probable that these small motile cells may prove better indicators of some of the reactions involved in fertilization than the slowly reacting egg.

There are three categories of behaviqr exhibited by spermatozoa that seem to me of importance for the problem of fertilization, because all are exhibited in reponse to egg secretions. These are (1) activation, (2) aggregation, (3) agglutination. The phenomena of activation are involved in those conditions that affect the activity of spermatozoa. The phenomena of aggregation are positive taxic responses, for the most past chemotactic. The phenomena of agglutination are exhibited in the presence of substances that cause the spermatozoa to adhere in masses. In a preliminary paper I have described some aspects of these phenomena (Science, N. S., vol. 36, October 18, 1912). We may consider the subject matter under these three heads.

The spermatozoa of marine animals in which fertilization takes place in the sea-water offer advantages for study probably greater than those of any other forms, because the conditions of normal activity are given in the sea-water itself, no secretions of accessory glands either of the male or of the female being requisite. Moreover, the spermatozoa may be obtained in large quantities. They offer, thus, material directly accessible to experimental work with the simplest possible facilities. Among the forms available for work, Nereis and Arbacia were soon found to be best adapted because the breeding season extends through most of the summer and they furnish material in large quantities. The present paper is therefore confined almost exclusively to these forms.

Suspensions of the spermatozoa in sea-water formed the material for all of the experiments. The reactions vary somewhat according to the density of the suspensions, and it may be important in future experiments to find some quantitative method of expressing the variations in density. But for the purposes of this paper it will be sufficient to indicate the extremes as opalescent, milky and creamy, with intermediate qualifications. An approximation to uniformity was attained in many of the experiments by adding a certain number of drops of the dry sperm[1] to measured quantities of sea-water.

[1] By 'dry sperm' is meant the sperm as it comes from the testes without the admixture of fluid.

II. ACTIVATION PHENOMENA

A. NEREIS

1. The aggregation reaction

We may begin the discussion of the behavior by describing a phenomenon which was used throughout the experiments with Nereis as a test of the activity of the spermatozoa. A drop of dry sperm from a mature Nereis is mixed in about 6 cc. of sea-water in a Syracuse watch crystal, making a uniformly milky suspension; in a few seconds clouds begin to appear and in fifteen to forty-five seconds these usually draw together in dead-white solid-looking masses uniformly spaced throughout the fluid. The intervening fluid becomes quite clear and the masses quickly settle on the bottom. The rate of formation of these masses, their number and size, depend on temperature, 'freshness' of the sperm and other conditions discussed beyond. Any sperm suspension that exhibits the aggregation phenomenon will be called 'aggregative' in the experiments that follow.

The appearance is of course due to the aggregation of the sperm in closely packed masses. Under a low power of the microscope each mass appears like a swarm of bees, owing to the intense activity of the peripheral spermatozoa. But those in the interior of the dense mass must be quiescent or the masses would break apart. After the aggregations have settled to the bottom of the crystal they tend to flatten out and may run together in time to a greater or less extent.

If, immediately after settling of the aggregations, the sperm is mixed up with a pipette, a perfectly uniform milky suspension is again produced, which may aggregate a second time, but more slowly than the first time, and in fewer aggregates; and the intervening fluid remains quite opalescent, showing that all the spermatozoa have not joined the aggregates. A third mixing up is not usually followed by aggregation until after the spermatozoa have settled to the bottom, and then only a very partial aggregation results.

A considerable number of variations of this theme can be produced by using sperm suspensions of varying density contained

in vessels of varying form, et cetera; under certain conditions the aggregations may arise in conformity with the water currents set up by the last emptying of the pipette, et cetera. But a description of these variations would be useless without the analysis of the causes of the phenomenon, which is taken up later.

All the experiments on Nereis to be described beyond were made with aggregative sperm, so that there was always a test, which had the advantage of being macroscopic and quick, of the activity of the sperm used in the experiments, and this has much to do with uniformity of results.

To give a more concrete idea I reproduce three photographs, natural size, of the phenomenon of aggregation. The first (fig. 1) was taken ninety seconds after mixing a drop of dry sperm in about 8 cc. of sea-water. The aggregations are quite uniformly distributed except in the upper right quarter where their arrangement marks out original currents produced by mixing with the pipette. Figure 2 was likewise taken ninety seconds after mixing; the effect of water currents on the arrangement of the aggregations is shown here quite well on the left. Figure 3 was taken three minutes after mixing, and the separate aggregations are beginning to fuse together on the bottom.

I propose to discuss in this section simply the conditions which modify the activity of the spermatozoa. In the case of Nereis such conditions may be inferred from two kinds of observations, namely: (1) The appearance of activity presented to the eye under the microscope and (2) the rate and degree of the aggregation reaction which is macroscopic. Nereis is the only form with which I am familiar that exhibits the latter reaction in any marked way. Its sperm is therefore better adapted than that of any other species for study of conditions of activity. The observations of different samples of sperm under the microscope are very difficult to compare as to degree of activity, as one is never sure of the successive subjective impressions, but in the case of Nereis these can be checked by the aggregation reaction.

The principle conditions that affect activity are 'freshness,' temperature, and the chemical constitution of the medium. These conditions will be considered not exhaustively at all, but

Fig. 1 Photograph of aggregation of a Nereis sperm suspension, taken 90 seconds after mixing the suspension; natural size; description in text.

Fig. 2 Another suspension photographed at 90 seconds; description in text.

Fig. 3 Another suspension photographed 3 minutes after mixing; description in text.

only to the extent that they appear to be significant for the phenomena of aggregation and agglutination, which are the main problems for our consideration.

2. Individual movements of the sperm

To explain the various reactions of the sperm it is necessary to consider first some of the more obvious features of locomotion of individual spermatozoa. In their free movements through the water they describe, as is well known, spiral paths. In Nereis the successive turns of the spiral are rather close set. As soon as a spermatozoon comes in contact with a surface, the movements of translation cease, and circus movements begin. The sperm moves round and round in a circle of varying diameter in contact with the surface. In the case of a preparation beneath a cover slip on a slide, those in contact with the slide rotate anticlockwise, those in contact with the cover-slip clockwise. The direction of rotation is always the same. It is associated no doubt with structural asymmetry which I described briefly in Study III (Lillie

'12). The tail of the spermatozoon is attached not to the center of the middle piece, but on one side.

The movement is of course due to successive beats of the tail and it is a very interesting fact that under certain conditions of aggregation the successive beats of the spermatozoa forming an aggregation may become synchronous, and under such circumstances the number of beats approximates 120 a minute at temperature of 21°C., if the sperm be fresh. This phenomenon, which I have not yet attempted to analyze in detail, follows after the aggregations of sperm from a fresh suspension have settled on the bottom of the container and begin to spread out of their own weight. Its appearance may be accelerated by gentle agitation of the dish, which tends to spread out the aggregations. Synchronous movements appear when the sperm spread out in a kind of membrane from an aggregated condition. In such a case the synchronous beats spread over the membrane thus formed, like waves of contraction over a ciliated epithelium. In fact, a kind of synthetic ciliated epithelium is then established. The interest of the phenomenon in the present connection is that it furnishes a clear demonstration of the successive beats of the tail of the spermatozoa, which are not readily distinguishable, and certainly cannot be counted, in the case of a single spermatozoon.

The movements of the spermatozoa are, then, due to successive beats of the tail, which is so placed as to cause rotation in a definite direction. The movement when freely suspended in water is in a spiral path, but when in contact with a surface the translatory component of the locomotion is almost entirely eliminated.

The following account of the behavior does not deal directly with individual movements, but always concerns mass-reactions, from which the behavior of the individual spermatozoa may be inferred.

3. 'Freshness'

The spermatozoa are absolutely immotile while they are in the body of the male, but become intensely active when suspended in sea-water. This expresses itself in the formation of aggregations; but, as already noted, aggregations form more slowly after

a second mixing up, and only to a slight extent or not at all after a third mixing. This condition of relative inactivity, or staleness, is reached in a few minutes, but varies more or less according to the density of the suspension, a very dense suspension exhibiting it more quickly than one less dense. The activity of the sperm may be restored, partly at least, by the addition of fresh sea-water, which shows that the staleness is not due to exhaustion, but to the accumulation of by-products of activity in the sea-water. Of these the chief is probably CO_2, as will be shown by experiments described beyond. The formation of CO_2 by the activity of the spermatozoa themselves is indeed one of the chief causes that limits their activity when sufficiently concentrated to form milky suspensions. To obtain the best results with the experiments described it is necessary to work with fresh sperm; otherwise, the accumulation of CO_2 may obscure the reactions.

4. Temperature

In 1911 a series of observations were made on the effect of temperature on the aggregation reaction of fresh sperm. In general the results as tabulated are:

13°C. No aggregations form
15°C. Slight signs of aggregation in 4 minutes
18°–19°C. Aggregation in from 2 to 4 minutes; much fewer in number than at higher temperatures
20.5°C. Aggregations, numerous, in 1 minute
23.5°C. Aggregations, yet more numerous, in 30 seconds
26.5°C. No aggregations form at this temperature, but they form as the temperature falls to 23°C.

In general temperatures from 20° to 23.5°C. are optimum for the aggregation phenomenon. At 15°C. the movements of the spermatozoa are too slow, and at 26.5°C. the movements are extremely active, but apparently uncoordinated, so that the aggregation reaction is not given. These figures possess no absolute value, but they indicate approximately the limits of temperature within which the reaction may be expected. The normal temperature of the sea-water varies from about 18° to 22°C. at Woods Hole during the breeding season.

5. Chemical composition of the medium

The effect of the chemical composition of the medium upon the activity of the spermatozoa is a very complicated subject, and no attempt has been made to analyze it farther than was necessary for comprehension of the forms of behavior studied. Even the simplest experiments furnish convincing proof of the dependence of activity of the spermatozoa upon a constant chemical composition of the medium; and this extreme susceptibility is certainly a prime factor in the behavior of the spermatozoa. To determine something of its limits becomes therefore necessary.

One of the first questions that presents itself is obviously the relation of the activity to the various salts of the sea-water. This is, however, in itself a problem of so much complexity that I have hesitated to undertake it; especially as it is unnecessary for our present purpose, seeing that the behavior to be studied takes place in sea-water as its medium. The few observations made demonstrate that spermatozoa of Nereis are paralyzed in pure M/2 solutions of NaCl, KCl, $CaCl_2$ and $MgCl_2$. As these are the principal salts of sea-water, it is obvious that the activation of the spermatozoa in the sea-water is a question of balance of salts. I therefore tried Van't Hoff's solution, namely: 100 cc. M/2 NaCl + 2.2 cc. M/2 KCl + 2 cc. M/2 $CaCl_2$ + 12 cc. M/2 $MgCl_2$, but the spermatozoa did not activate in this solution either. Some other experiments were made, which did not materially help the problem, which was not followed farther. The later experiments all assume the sea-water as the given medium.

Some early observations in the course of this work had shown that the female excretes certain substances in the sea-water that have a strong inhibiting effect upon the activity of the spermatozoa. This is so marked that sperm suspensions made up in sea-water sufficiently charged with secretions of the females never exhibit the aggregation phenomenon, and their fertilizing power is markedly reduced. This fact repeatedly observed suggested tests on the susceptibility of the spermatozoa to CO_2 dissolved in the sea-water, and this formed the beginning of a series of tests that involved acids and alkalis and some other substances.

a. Susceptibility to acids, including CO_2. The susceptibility of spermatozoa of Nereis to acids was tested by opening a male Nereis in a dry watch crystal, and mixing a drop of the thick sperm, which flows out, in 6 to 8 cc. of the solution to be tested. The effect on the movements of the spermatozoa was then observed as rapidly as possible, first with the naked eye to control the aggregation reaction which is given only by very active spermatozoa, and second with the microscope to note the degree of activity if their movements were sufficiently slowed down to prevent the aggregation reaction. For each experiment a control suspension of spermatozoa in normal sea-water was run, and only those experiments are taken into account in which the control aggregated in ninety seconds or less. The acid solutions were made by adding a sufficient quantity of N/10 dilutions in distilled water to a measured quantity of sea-water to reach the dilutions tested. These were always so weak as not to involve the question of osmotic changes in the results. The results may be tabulated as follows:

	H_2SO_4	HCl	HNO_3	$CH_3.COOH$
N/1000	0	0	0	0
N/2000	1	1−	1	0
N/3000	2	2+	1	0
N/5000	3	4	3−	1
N/10000	4	4	4	4

In this table (0) stands for complete paralysis
(1) represents minimum amount of movement; usually only a few spermatozoa moving
(2) Fairly active, but no aggregations form
(3) Active; aggregations form, but are few in number and require over two minutes to appear
(4) represents maximum activity, aggregations forming at least as rapidly as in the control, i.e., in less than ninety seconds

While the four grades of activity noted are readily to be distinguished in Nereis, their relation to the grades of acidity in question is not to be taken as fixed and invariable. As a matter of fact the various observations show considerable variation with reference to the intermediate dilutions, N/2000 to N/5000, in the case of the mineral acids. But in the case of the extremes N/1000

always produces complete paralysis very quickly, and N/10000 always permits maximum activity. The range of activity with reference to these acids is thus marked out fairly well. It will be noted that acetic acid has a greater inhibiting effect than the mineral acids.

It is an interesting question in this connection whether there is a certain optimum amount of acidity which increases rather than decreases the activity of the spermatozoa. In the experiments now under consideration this could not be determined certainly. In some cases spermatozoa aggregated more rapidly in weakly acid solutions (N/5000 and under) than in the control; in others at the same rate or at a slightly less rate. In the experiments on chemotaxis, however, which involve an acid gradient, there is possible evidence of stimulation of weak solutions.

CO_2. The sensitiveness of the spermatozoa to CO_2 is considered separately because of its probable biological significance and also because it was impracticable to state the strength of the solution in molecular terms. The solutions were prepared empirically as follows: A certain quantity of sea-water was supersaturated with CO_2 in 'Sparklet' siphons. The charged sea-water was drawn as desired, and after the effervescence ceased it was diluted with measured quantities of sea-water, and the dilutions were expressed as percentages of the charged sea-water. These solutions were always prepared fresh for each experiment, and kept in stoppered bottles or otherwise covered as far as possible. The uniformity of the reactions obtained is adequate proof that the solutions used in the different experiments were equivalent.

A very large number of experiments was made with CO_2 during the course of the summer, so that the relations of the spermatozoa to CO_2 were more adequately ascertained than for any other substance. Here the question is only of the relation of CO_2 tension to the activity of the spermatozoa, and the results may be stated as follows:

One per cent of the charged CO_2 sea-water paralyzed the spermatozoa immediately; or rather a suspension of a drop of dry sperm in 6 to 8 cc. of this strength of CO_2 does not exhibit any activity. This is, however, very near the minimum paralyzing

dilution, and some samples of sperm will exhibit slight movements in it.

In 0.75 per cent CO_2 sea-water no aggregations take place, but the spermatozoa move feebly.

In 0.5 per cent CO_2 sea-water aggregations usually form slowly, but the activity is usually less than the control.

In 0.33 per cent CO_2 sea-water there is apparently no inhibition of activity as compared with the control.

Whether lower dilutions stimulate more than normal sea-water is difficult to say by the method used here. But the chemotaxis experiments possibly indicate stimulation at a certain optimum (see p. 535).

The sensitiveness of Nereis spermatozoa to CO_2 is thus surprisingly great, and it operates within very narrow limits. This is the more surprising when comparison is made with spermatozoa of other species. Thus I ascertained that the sperm of Loligo will move, though feebly, in 50 per cent CO_2-charged sea-water, and that it is very active in 20 per cent, though less so than in normal sea-water. In the case of Chaetopterus it requires about 33⅓ per cent to 40 per cent of the CO_2 sea-water to completely paralyze all the spermatozoa, though 10 per cent inhibits considerably. Arbacia sperm on the other hand is much more sensitive to CO_2, being completely paralyzed in 3 per cent. But Nereis is very much more sensitive than any of these, and this involves some very interesting forms of behavior described later on.

b. Sensitiveness of spermatozoa of Nereis to alkalis. Alkalis above a certain concentration agglutinate the spermatozoa of Nereis, and cause them to stick together in masses. This is never seen in acids, however strong. I can best state the sensitiveness of the spermatozoa to KOH by giving the protocol of a single experiment (June 23, 1912) which followed some preliminary determinations. N/10 KOH in distilled water was used as the standard solution. Added to sea-water this solution produces a precipitate which redissolves up to about N/2500 KOH.

In the experiment a drop of dry sperm was stirred in about 8 cc. of each of the following dilutions in sea-water, and observations made as noted.

1. N/2500 KOH. Produces very rapid agglutination of the spermatozoa; free sperm between show some movement.
2. N/5000 KOH. No agglutination; no aggregation; sperms fairly motile.
3. N/7500 KOH. No agglutination; no aggregation; sperms more active.
4. N/12500 KOH. No agglutination; no aggregation; sperms very active.
5. N/25000 KOH. No agglutination; aggregations form slowly; but sperms are extremely active.
6. Normal sea-water. Control. Aggregations form in half minute. Very active sperm (maximum).

This experiment was carried out with the sperm of one male at one time, the solutions being prepared in advance. The limits of the agglutination effect are given. But it is improbable that the inhibiting effect extended to the lower limit, although aggregations were formed so slowly in N/25000. The reason for this, as will be shown later, is that the aggregation effect is due to positive chemotaxis to a weak acid, probably CO_2, produced by the spermatozoa themselves. This is neutralized by the KOH so that in spite of the great activity noted in N/25000 KOH aggregation cannot take place until after neutralization of the alkali.

If the behavior of the spermatozoa be observed under the microscope at the moment they are put into N/2500 KOH, there is seen momentary great activity of the spermatozoa followed quickly by agglutination as described.

The relations to NaOH were essentially the same. There was slight agglutination in N/5000 NaOH, and the slightest appearance of aggregations in N/25000 NaOH.

c. To alcohol and ether the sensitiveness is as follows:

Alcohol:

(1) 5 per cent, sperm are paralyzed
(2) 2 per cent, some activity; no aggregations
(3) 1 per cent, more active, some aggregations may form in five minutes
(4) 0.5 per cent, few aggregations in about three minutes
(5) 0.2 per cent aggregations in forty-five seconds
(6) 0.1 per cent, aggregations in thirty-two seconds
(7) Control, sea-water; aggregations in thirty seconds

(4), (5), (6) and (7) came from an experiment with the same sample of sperm.

The sensitiveness to ether is essentially the same, though the sperm did not aggregate even at 0.33 per cent. The chemotaxis experiments with ether indicate a possible stimulation of the sperms at an optimum concentration (see beyond).

As stated before, no attempt was made to carry the analysis of the relation of activity of the spermatozoa to known chemical substances very far. Experiments on chemotactic and other behavior phenomena of the spermatozoa were in progress at the same time, and the determinations already given seemed fairly adequate for the purposes of analysis.

6. Sensitiveness of spermatozoa to hypo- and hyper-tonicity of the medium

As regards the sensitiveness of spermatozoa to hypo- and hypertonicity of the medium, the following determinations may suffice:

August 18, 1911. Sperm of one male; one drop mixed in each of the following solutions, with results noted:

(1) 5 cc. sea-water + 2.5 cc. distilled water. The sperm are fairly active, but no aggregations form.

(2) 5 cc. sea-water + 1 cc. distilled water
(3) 5 cc. sea-water + 0.5 cc. distilled water
(4) 5 cc. sea-water
} Aggregations form in one minute; a little better in 4

(5) 5 cc. sea-water + 1 cc. 5/2 N NaCl; sperm paralyzed

The spermatozoa will thus stand considerable decrease in osmotic pressure without much modification of activity. But increase in osmotic pressure induced in the experiment by addition of NaCl and in others by KCl, $CaCL_2$ or $MgCl_2$, rapidly paralyzes. The addition of this amount of KCl paralyzed every sample of sperm used and its effect is undoubtedly toxic; but some samples of sperm exhibited considerable, though decreased, activity, when the other salts were used.

B. ARBACIA

The tests concerning the relation of the activity of spermatozoa of Arbacia to the chemical composition of the sea-water were not so extensive as in the case of Nereis. Moreover we do not have any definite aggregation reaction here to serve as a measure of activity. However, a sufficient number of tests were made to show that Arbacia is much less sensitive to variations in inorganic constituents than Nereis.

CO_2. To afford comparisons we may use three forms here, Chaetopterus, Arbacia and Nereis:

	100% CO_2	40% CO_2	20% CO_2	10% CO_2	5% CO_2
Chaetopterus	paralyzed	few move slightly	many move	active	active
Arbacia	paralyzed	paralyzed	paralysed	paralyzed	paralyzed
Nereis	paralyzed	paralyzed	paralyzed	paralyzed	paralyzed

	2.5% CO_2	1% CO_2	0.5% CO_2	NORMAL SEA-WATER
Chaetopterus	active	active	active	active
Arbacia	traces of movement	fairly active	active	maximum activity
Nereis	paralyzed	paralyzed	fairly active	maximum activity (aggregation)

Thus, while compared to Chaetopterus, Arbacia, is extremely sensitive to the presence of CO_2, compared to Nereis it is relatively insensitive.

To other acids, H_2SO_3, HCl, HNO_3, and CH_3COOH, Arbacia is also less sensitive than Nereis, exhibiting a fair degree of activity in N/1000 solutions in sea-water (compare table for Nereis, p. 526).

The sensitiveness to alkalis does not differ materially from that of Nereis. Agglutination of the spermatozoa is caused by N/2500 KOH, giving a very pretty precipitation picture in a vial. Such agglutinations are irreversible. Spermatozoa between the agglutinated masses may be in motion.

The relatively slight sensitiveness of Arbacia sperm to CO_2 is correlated with absence of any such striking aggregation effects

as are exhibited by suspensions of Nereis spermatozoa. But indications of the same kind of reaction may be seen under certain circumstances. Thus, a fresh suspension mounted beneath a raised cover will soon exhibit cloud effects due to differences in the density of aggregation, and this corresponds to the first stage of aggregation in Nereis. In the course of an hour or so all the spermatozoa retract from the edges into a central dense aggregation, and this is due, I believe, to the rising CO_2 tension towards the center. Reasons for this opinion are given under the head of the aggregation phenomena.

Fig. 4 Reaction of spermatozoa of Nereis to a drop of 1 per cent CO_2 sea-water; from an experiment of June 18, 1912. The original sperm suspension was made at 3.09; it aggregated clearly at 3.10 (fig. 1). It was then mixed up with a pipette and some drops mounted on a slide beneath a raised cover-slip as in *1*. The drop of 1 per cent CO_2 sea-water was introduced at 3.12¾ (on left) and a drop of pure sea-water as control (drop to right). Figure 4, *1*, shows the reaction at 3.13; *2*, at 3.14; *3*, at 3.14½, and *4*, at 3.16. In *4* the general suspension has aggregated. The final position of the reactive sperm is in the center of the introduced drop. No reaction takes place with reference to the drop of sea-water, which gradually becomes obliterated by inwandering of sperm. The figure shows also that the spermatozoa retract from the margin of the suspension.

III. AGGREGATION PHENOMENA

The spermatozoa of Nereis and Arbacia show very definite positive chemotaxis toward acids and egg-extracts of the same species, which may be demonstrated with striking clearness by the method first introduced by Jennings in studying the behavior of Paramecium. The method as applied to behavior of spermato-

zoa consists in mounting some drops of a sperm suspension beneath a long cover slip supported by glass rods, and injecting a drop of the fluid to be tested into the suspension. It then forms a clear drop within the milky suspension, and reaction at once begins at its borders. This method gives incomparably more delicate results than Pfeffer's method of using capillary glass tubes. The drop is confined above by the cover and below by the slide and diffusion takes place only at its margins; in this way a gradient is established. In the case of the capillary glass tubes diffusion is so slight from the open ends that no delicate reaction can be expected. So after a number of trials the capillary tube method was abandoned and the injected drop method was used exclusively.

A. NEREIS

1. Aggregation with reference to CO_2

As introduction, the reaction of a fresh suspension of the spermatozoa of Nereis to a 1 per cent dilution of sea-water saturated with CO_2 will first be described from a specific experiment:

June 26, 1912. A ripe male Nereis was placed in a dry watch crystal and snipped with scissors; two drops of the dry sperm were mixed in 10 cc. sea-water at 9.11½ A.M. and made a milky suspension which aggregated freely in thirty seconds. Some drops of this were then mounted beneath a cover slip supported by glass rods about 1 mm. in diameter. A drop of the 1 per cent CO_2 sea-water was injected at 9.13. In withdrawing the pipette a trail of the CO_2 sea-water is left extending to the margin. In a few seconds the following configuration developed (fig. 4–*1*). It consists essentially of a dense aggregation of very active spermatozoa in the form of a ring within the margin of the original drop, and a line extending from the drop to the edge where the pipette was introduced and withdrawn. In this case the ring is open below and a linear aggregation extends from the opening towards the margin of the suspension. The ring and the linear aggregation are separated from the general sperm suspension by a clear area devoid of spermatozoa 1.5 to 2 mm. in width. This area

belonged mainly to the original territory of the sperm suspension, and the ring owes its origin to migration of spermatozoa towards the drop. They do not, however, penetrate at first to the center of the drop, but their movements are arrested, hence the formation of the ring. The 'tail' of the ring is due to migration of spermatozoa to the trail of CO_2 sea-water left behind in withdrawing the pipette, leaving a clear zone marking the range of the effective stimulus. Control: No reaction is given to a drop of pure sea-water similarly introduced.

The migration of spermatozoa to the first formed ring continues for a short time; the ring thus grows broader and tends to close in the center (fig. 4–*2* and *3*). Shortly after the ring and tail aggregations have formed with reference to the introduced drop of CO_2 sea-water, the usual aggregations of the sperm, 1 to 2 mm. in diameter, form in the remainder of the suspension outside the drop evenly spaced throughout, if the sperm suspension is perfectly fresh (fig. 4–*4*). But if it is a little stale the general suspension remains homogeneous.

The detail of form of the ring and tail aggregations vary according to whether the introduced drop simply displaces a certain amount of the suspension, or is more or less mixed in the introduction; and this depends obviously on the size of the opening of the capillary pipette and the rate at which the drop is introduced. But the general form of the reaction is always the same.

The spermatozoa in the CO_2 aggregations are never in the least agglutinated and their behavior is in all essential respects the same as in the aggregations formed in any fresh suspension. I therefore early formed the hypothesis that the aggregation phenomenon is a chemotactic reaction to CO_2 produced by the spermatozoa themselves, and this hypothesis has been abundantly confirmed, as the series of experiments to be described will show.

The formation of the described configuration in a suspension of active spermatozoa with reference to an introduced drop is due to positive chemotaxis to the drop. If the clear margin be observed during the formation of the ring, the spermatozoa may be seen swimming across it to the ring head first. Under the low power of the microscope they appear to drift across it with a

dancing motion like motes in a sunbeam owing to their spiral path. If the external edge of the clear zone be carefully observed, the spermatozoa can be seen to detach themselves one by one from the general suspension and pass straight over to the ring. But only those freely suspended make the direct path; those in contact with the slide or cover continue their circus movements; the chemotactic stimulus seems unable to overcome the thigmotactic reaction.

The reaction is given most clearly and rapidly by a fresh sperm suspension, although one which has passed the aggregation stage still gives it; however, as the sperm suspension becomes stale the reaction becomes slower, and eventually ceases. Spermatozoa killed by gentle heat give no such reaction, thus excluding any purely physical diffusion effect as cause of the phenomenon.

In the case of this reaction in a somewhat stale non-aggregative suspension the movements of the spermatozoa on the outer margin of the ring are decidedly more vigorous than in the general suspension. This would appear to indicate that, at this place in the CO_2 gradient marked by the clear zone, the concentration of the CO_2 is stimulating rather than depressing; but when we consider that the CO_2 gradient must rise from the suspension across the clear zone to the ring, and that the relative inactivity of the sperm in the suspension is due, partially at least, to CO_2 the conclusion is not so clear. In any event, if we attribute a stimulating action to a given CO_2 concentration on such evidence, we must regard the depression of activity in the general suspension as due partly to other excreta.

The conditions established by the experiment may be represented diagrammatically as follows (fig. 5). The injected drop is represented by the continuous line circle and continuation, the general suspension by the shaded area. By diffusion from the injected drop a CO_2 gradient is established outwards, and this must extend into the drop a certain distance because the gradient is established by loss of CO_2 from the drop. The concentric broken lines represent the gradient, or at least that part of the gradient which is affective in the reaction. The thick open circle and the similar linear extension represent the aggregations of the sperma-

tozoa. At the same time there is of course diffusion of substances peculiar to the general suspension towards the introduced drop; but that conditions thus arising are ineffective is shown by the fact that no reaction is given to the introduced drop of pure sea-water. We may, therefore, leave this centripetal diffusion out of account. It should be remembered that 1 per cent CO_2 sea-water is the minimum paralyzing strength for Nereis sperm.

Fig. 5 Diagram of the reaction of a sperm-suspension of Nereis to an introduced drop of 1 per cent CO_2 sea-water; explanation in the text.

The diagram therefore shows, that migration of the spermatozoa proceeds up the gradient to, or near to, the point of paralysis of the spermatozoa; for in the case of the drop of 1 per cent CO_2 sea-water the ring forms well within the original margin of the drop. With higher and lower dilutions of CO_2 the width of the clear margin is practically the same.

The effects of greater and less CO_2 concentration than the 1 per cent used in the initial experiment are interesting. In general the use of a greater concentration involves a larger aggregation, and of a less concentration a smaller aggregation. Thus if a drop of sea-water saturated with CO_2 be introduced into a suspension of fresh sperm beneath a raised cover slip a border of dead or paralyzed sperm forms at its margin, and shortly a clear zone forms external to it; the spermatozoa migrate in large

numbers across the clear margin to the ring and are speedily paralyzed by the diffusing CO_2; this process continues very rapidly and as a consequence the central aggregation expands, and may in time absorb all the spermatozoa of the suspension.

The same phenomenon in less pronounced form is exhibited by the reaction to a 1/10 dilution of the saturated CO_2 sea-water. Here we may give a definite experiment with measurements of growth of the aggregation: July 1, 1912. Into a fresh sperm suspension beneath a raised cover a drop each, (a) of 1 per cent, and (b) of 10 per cent CO_2 sea-water was injected some distance apart. Drop (a) measured 5 mm. in diameter and drop (b) 3 mm. immediately after injection at 2.28 P.M. The aggregations caused by these drops measured at 2.32: (a) 3 mm. (b) 5 mm. That is to say, the aggregations formed *inside* the drop in case of the weaker solution, and outside in the case of the stronger. At 2.35 (a) still measured 3 mm. and (b) now 6 mm. In a repetition of this experiment drops (a) and (b) each measured 3 mm. at 2.37. The aggregations caused by them measured at 2.40, (a) 2 mm., (b) 5 mm.; at 2.47, (a) 2 mm., (b) 10 mm.

It is clear from these observations that the spermatozoa are positively chemotactic in a CO_2 gradient where the tension is above a certain point, and that the aggregation caused by the more concentrated drop grows because the diffusion of CO_2 from the center forms a widening ring of the necessary concentration. To furnish a gradient the concentration must exceed the CO_2 tension in the general suspension, which is a function of the age of the sperm suspension and the activity of the spermatozoa in it, and on the other hand a limit is set to the differential which furnishes the reaction by the fact that a concentration of about 1 per cent of the saturated CO_2 sea-water paralyzes the spermatozoa. The gradient that furnishes the reaction must, therefore, operate within very narrow limits.

Greater dilutions of the CO_2 sea-water than 1/100 will act positively in the case of fresh sperm suspensions. The ring forms within the margin of the drop in the case of 1/200 dilution, but it remains narrow, and tends to break into bead-like aggregations, proving that the spermatozoa by their own activity have produced

a greater CO_2 concentration within the ring, which furnishes centers of aggregation positive to the 1/200 concentration of the drop. Drops below this concentration or drops of pure sea-water furnish no reaction.

Summarizing; it would appear that the spermatozoa of Nereis follow a CO_2 gradient to the point of paralysis (about 1/100 saturation). The clear zone outside an aggregation represents the effective CO_2 gradient in every case. The various forms of reaction to drops of different concentrations follow from this simple principle.

2. Interpretation of the aggregation reaction

We are now prepared for the interpretation of the aggregation phenomena exhibited by fresh sperm suspensions described on page 519. The spermatozoa as they come from the body cavity are absolutely quiescent; as soon as they are suspended in sea-water they become intensively active, and consequently produce CO_2 very rapidly. Any area of greater concentration of spermatozoa, by producing more CO_2 than other areas, becomes a center of attraction, and aggregations of the spermatozoa once begun are bound to proceed to the limit, because the closer the aggregation the greater the CO_2 production and consequently the greater the chemotactic stimulation. If aggregations once formed are broken up and the spermatozoa evenly suspended once more, the CO_2 tension in the suspension is greater than at first and is evenly distributed. Hence, in the first place the activity of the spermatozoa is reduced, and, in the second place, the differential of the gradient between that of the general suspension and the point of paralysis is greatly lessened. Therefore aggregation takes place more slowly and less completely than before; and, after a second and a third stirring up, the CO_2 tension in the entire suspension has become too great to permit of sufficient activity to react to the slight possible differential gradient.

It is obvious that such a reaction can take place only in the case of spermatozoa that exhibit extreme sensitiveness to CO_2. The spermatozoa of Nereis possess by far the greatest sensitiveness to CO_2 of any studied, as we have already seen. No other

spermatozoa exhibit the aggregation reaction in any marked form so far as I know; Arbacia which comes next to Nereis in point of sensitiveness to CO_2 among the forms studied, shows, under certain conditions, a cloud-like formation similar to the initial stage of aggregation in Nereis. These will be referred to beyond.

It may perhaps be objected that the aggregation reaction in Nereis is not necessarily caused by CO_2 excretion, but possibly by some other substance produced by the spermatozoa. And it would be difficult to meet this objection in any absolutely conclusive way. But the following considerations render the conclusion extremely probable. In the first place it can be proved that the spermatozoa exhibit positive chemotaxis towards some substance that they themselves produce. Thus July 31, 1912, the following experiment was made:

With the dry sperm of one individual two suspensions were made at the same time (8.57 A.M.) namely: *a* one drop of sperm in 9 cc. sea-water, *b* two drops of sperm in 6 cc. sea-water; the activity of the sperm in both suspensions being the same *b* should produce any attractive substance in much greater amount than *a*. This was tested by making preparations of *a* and *b* on separate slides beneath raised covers. A drop of *b* was then injected into slide *a*, and a drop of *a* into slide *b* at 8.59. On slide *a* there was a very quick beautiful positive reaction to the introduced drop, that is a clear border formed about the drop owing to positive chemotaxis of the spermatozoa *a* to the drop of denser suspension *b*. On slide *b* not only was such positive reaction absent, but the drop of introduced sperm actually lost its spermatozoa and became clearer, owing to the positive chemotaxis now being away from the drop. The same results were obtained also by using two suspensions of equal density, one of which was older than the other. The fresher suspension reacted positively to the older suspension.

In the second place, it is of course certain that the spermatozoa becoming suddenly active in the sea-water must produce CO_2; and as we have seen that the spermatozoa of Nereis react even to a 1/200 dilution of a saturated solution of CO_2 in sea-water, if it can be proved that a standard suspension of spermatozoa produces an equivalent amount, the probability that CO_2 is the agent involved in the aggregation effect become very great. Experiments directed to this end showed that a 1/100 dilution of CO_2

lies near the limit of demonstrability both by color reaction tests and also by gas-burette estimation. A number of tests of sperm suspensions were made with acid color indicators. In the case of neutral red a dilute solution in sea-water has a decided orange tinge due to slight normal alkalinity of the sea-water. The same dilution made from a standard concentrated solution by the addition of a sperm suspension, shows a decided rose color without any trace of orange. The spermatozoa then aggregated in the vial used and the aggregations sank to the bottom, forming a bright red precipitate, and the supernatant fluid, now merely opalescent on account of the few sperms remaining in it, was faint rose. There is thus a decided acid reaction of the sperm suspension. Tests with azolitmin and tropaeolin 000 No. 1 also gave clear indications of acid. The sperm suspensions were tested within two minutes or less after their preparation; the liberation of the acid takes place therefore very suddenly. It is liberated only when the sperm become active, and the change of color is not given if the sperm remain inactive. It is therefore very probable that CO_2 is the acid revealed.

Finally a large number of tests for CO_2 were made of the air in closed flasks containing considerable quantities of active sperm suspensions of Arbacia. The details of these tests made with a gas burette need not be given. They extended over a week, using the sperm of Arbacia which could be obtained in larger quantities than Nereis. Although the determinations came very near the limits of experimental error, there could be no question as to the presence of CO_2 in quantities above that contained in the air or in normal sea-water.

In consideration of the facts (1) that sperm suspensions of Nereis produce a substance to which spermatozoa of Nereis react positively (2) that an acid is present in the suspensions (3) that the production of CO_2 by the suspensions can be demonstrated and (4) that spermatozoa of Nereis react positively to dilutions of CO_2 in sea-water which are barely detectable by color indicator, or gas burette, it can hardly be questioned that the aggregation reaction in Nereis is due to positive chemotaxis to CO_2.

3. Reaction to other acids

The sperm of Nereis exhibits the same positive chemotaxis to other acids as to CO_2. It is hardly necessary therefore to enter into details. Sulphuric, hydrochloric, nitric, and acetic acids were tested. They agree very closely with respect to the effective dilutions; N/1000 dilution of any of these acids is an effective chemotacticum agreeing quite closely in the degree of the effect produced with 1 per cent CO_2; N/2000 may cause slight ring formation in a drop introduced into a fresh sperm suspension, but, if the suspension has reached the non-aggregative stage, no reaction ensues, owing to the fact that the acid concentration in question furnishes no gradient.

Drops of stronger concentration cause a ring-shaped aggregation which continues to grow until diffusion eliminates the acid gradient. None of these acids cause the least sign of agglutination of the spermatozoa whatever their strength.

A drop of N/10 acid introduced within a sperm suspension beneath a raised cover kills all the spermatozoa in its immediate neighborhood, as the acid diffuses the zone of dead sperm increases but as the margin of the diffusing acid reaches a dilution that is no longer fatal it becomes marked by a clear border which is due to the migration of sperm to it, even though they are carried into a fatal concentration; and so the drop continues to grow so long as an acid gradient remains.

There is never the least sign of negative chemotaxis with respect to any concentration of any acid, nor indeed of any other agent tested. This being the case it is obvious that the aggregation of the spermatozoa can not be by any trial and error method of behavior, but must take place through orientation.

4. Behavior with reference to alkalis

The spermatozoa of Nereis do not exhibit any chemotactic reaction, positive or negative, to drops of KOH or NaOH injected into a suspension beneath a raised cover-slip. The drop remains empty at first, and spermatozoa that enter it by chance are agglutinated, so that in a short time the drop becomes filled

with agglutinated sperm masses. The alkalis were used in concentrations of N/2500 and N/5000.

Though no chemotatic reaction takes place, yet an interesting reaction may be procured by injecting a drop of N/5000 KOH into a sperm suspension made in 1/100 dilution of saturated CO_2 sea-water. The sperm in this are nearly or quite paralyzed, and will not of course react to an injected drop of 1/100 CO_2. But if a drop of N/5000 KOH be injected into such a suspension beneath a raised cover glass, motility of the spermatozoa returns at a short distance from the margin of the introduced drop, due evidently to neutralization of the acid, and aggregations of the active sperm may form outlining the KOH drop a certain distance from the margin. This experiment furnishes at once an interesting demonstration of recovery from CO_2 narcosis, and of the nature of the contrast between the acid and alkali reaction of the spermatozoa.

5. Reactions to other substances

It lay entirely outside of the scope of this work to attempt an exhaustive analysis of the behavior of the spermatozoa with reference to chemical substances. The investigation was undertaken to analyze the relations of the behavior of spermatozoa to fertilization; and when the principle of chemotaxis was once demonstrated, and the relation of this chemotactic reaction to the very striking aggregation reaction, it seemed better to turn at once to the subject of the behavior of the spermatozoa towards eggs and egg-secretions of the same species. It may be remarked incidentally that tests with alcohol and ether gave negative results, that is, no evidence of positive or negative chemotaxis was found. In some cases there was slight indication of ring formation near the margin of an introduced drop of 5 per cent alcohol or ether in sea-water, which may indicate a stimulating effect of the spermatozoa at an optimum concentration, but which, in the absence of a clear margin external to the ring cannot indicate chemotaxis.

6. *Thermotaxis*

The spermatozoa of Nereis do not exhibit any positive response to drops of sea-water at higher temperatures. Into suspensions of spermatozoa in sea-water at 21°C. under raised cover-slips drops of sea-water at 44°C., 52°C., and 84°C. were injected successively. No ring formation occurred with reference to any of these drops. They did not fill up with sperm as rapidly as drops of sea-water at room temperature, but this is no doubt due to the paralysis that sets in, as previously noted above about 28°C. The only observable effect of the heated drops was that aggregations formed a little earlier in the general suspension near the margins of the introduced drop; and this is attributable to increased activity of the sperm owing to rise of temperature, hence increased CO_2 production in the zone of increased activity; and more rapid aggregation as a consequence.

7. *Thigmotaxis*

In contact with any solid object Nereis spermatozoa tend to carry out circus movements in an anti-clockwise direction, when, fresh, but may soon come to rest. In any field of the microscope in a suspension beneath a cover glass one sees many of the spermatozoa in contact with the slide at rest, and many others carrying out the circus movements, while those that are freely suspended swim in spiral paths. The thigmotatic reaction then appears first to be the exaggeration of the rotation component of the ordinary locomotor movements, and second rest.

This reaction may of course come in conflict with the chemotactic reaction, as for instance in the clear margin external to the ring of spermatozoa produced in response to a drop of 1/100 CO_2 sea-water. Within this area all the freely suspended spermatozoa swim directly towards the ring, but those in contact with slide or cover-slip may continue their circus movements without any apparent directive effect from the CO_2 gradient. The thigmotactic stimulus appears thus to be more effective than the CO_2 gradient.

This thigmotactic reaction may be the starting point apparently of some of the aggregations formed in suspensions. Thus aggregations tend to form in the angle between the glass rod and the slide, which appear to owe their origin to the thigmotactic reaction; but when a considerable number of spermatozoa have accumulated in the angle their CO_2 excretion acts as a positive chemotactic stimulus on the sperm of the suspension, and a dense swarm soon forms along the rod, filling the angle and extending beyond it. Such a continuous swarm then tends to break into evenly spaced masses still in contact with the rod, owing to variations in CO_2 production. Thus thigmotaxis in this case is the initial cause of aggregations, which owe their subsequent growth to chemotaxis.

It may be that the thigmotactic reaction is a frequent cause of aggregations, particularly in suspensions that have produced considerable CO_2 when aggregations form only slowly and always in contact with the substratum. But in fresh suspensions this cannot be the case, for the aggregations first formed are freely suspended. In many cases aggregations may be seen to form with reference to firm strands or fibers of mucus in a suspension, and in such cases it appears probable that thigmotaxis and chemotaxis are combined.

8. Variations of reactions

So far as observed the behavior of sperm suspensions in sea-water may be quite fully explained by the forms of reaction described, and this brings the present section to a natural conclusion. But we may finally note certain variations of the reactions. The sperm suspensions were usually made, as stated, by mixing a drop or two of dry sperm with about 8 to 10 cc. of sea-water in a Syracuse watch crystal. This was done with a pipette, drawing in the suspension and squirting it out again until the sperm was evenly mixed. If this is done from one side, as is usually the case, a current is made across the dish to the opposite side and back along both sides, creating miniature whirlpools. Such currents of course come to rest in a few seconds, but when the aggregations become visible, ten to forty or more seconds later, they define very accurately the original currents. At first I thought natur-

ally of some rheotactic reaction. But on more careful examination and consideration the following explanation appears much more probable. Microscopic aggregations must begin to form while the water currents are still moving; they are then elongated by the friction in the direction of the current, and as they grow to macroscopic size the aggregations tend to preserve this form. The definition of the currents is due to the form of the aggregations rather than to their arrangement, and as they contract to spherical form the current-figures become less pronounced and very largely disappear.

Very interesting configurations may be produced in a sperm suspension of Nereis by dropping in dilute acids. In a few seconds quite complex wreath-like or festooned aggregations of spermatozoa appear at the site of the entering drop marking out accurately the distribution of the acid in the suspension. These of course vary with the strength of the acid, and the distance from which it is dropped.

If a few drops of a suspension of active Nereis sperm be mounted beneath a raised cover slip, it will be observed that the outer margin of the suspension for a width of 1 to 2 mm. soon becomes free from spermatozoa, thus tending to concentrate the suspension the same distance from the margin (fig. 4). This concentrated ring of the suspension then tends to form aggregations more rapidly than the more central parts. In the case of a suspension that is not perfectly fresh, aggregations may form only in this ring. The withdrawal of spermatozoa from the margin of the drop might at first thought be attributed to a negative chemotaxis towards oxygen. However, it is almost certainly not this, but a positive reaction towards the higher CO_2 tension of the interior of the drop. If a drop of sea-water saturated with oxygen be injected into a suspension beneath a raised cover, the spermatozoa avoid it in the same way that they do the free margin of the suspension.

The spermatozoa of Nereis make an acid indicator more delicate than any of the chemical dye indicators. In the course of some experiments I discovered quite accidentally, thus avoiding an awkward mistake, that the first few drops of water through any

available filter paper give a distinct acid test, using the spermatozoa of Nereis as indicator: A drop injected into a fresh sperm suspension invariably gave the ring formation with outer clear border, which is the characteristic and unmistakable acid reaction. This suggests the possible use of such cells as indicators in certain classes of experiments; some preliminary observations which I have made concerning CO_2 production of dividing eggs by this method are distinctly promising, though the results are complicated by the usual presence of other substances.

9. Chemotaxis to egg-secretions

The spermatozoa of Nereis exhibit positive chemotaxis to egg-secretions, which may be demonstrated in the same way as the positive chemotaxis to acids, but this subject, which is of course the most important part with reference to the fertilization problem, may be postponed to the next section dealing with agglutination phenomena, because it is always associated with agglutination.

B. ARBACIA

The reactions of Arbacia spermatozoa are essentially the same in principle as those of Nereis, but on account of the lesser sensitiveness of the spermatozoa, as noted in the section on activation phenomena, the reactions are much slower and less clearly defined. This may be illustrated by the notes on a single experiment: July 20, 1912. A fresh suspension of Arbacia sperm was made by mixing two drops of the dry sperm with 9 cc. of sea-water. The suspension appears milky, and the spermatozoa are decidedly active under the microscope. A portion was immediately mounted under a raised cover-slip and two drops of 5 per cent CO_2 sea-water were injected some distance apart. At first there appeared to be no reaction, as contrasted with Nereis in which ring formation is almost instantaneous under such circumstances. In two minutes the sites of the drops became more cloudy than the rest of the slide, and a faintly defined clear margin began to appear surrounding the drop. The picture gradually gained in definiteness until it became very clear. The central aggrega-

tions at first showed radiating strands of sperm visible to the naked eye, but then closed to form a solid drop, and this grew at its margins, preserving the clear external zone, until the clear margins of the two drops, at first several millimeters apart, ran together. Outside of the influence of the drops cloud formations appeared, corresponding to an early stage of the aggregations of Nereis.

The clear margin of the drops is not by any means so well defined as in Nereis. Moreover, the spermatozoa are so small that it is difficult to observe their behavior in the clear margin. However, there can be no doubt that the phenomenon is essentially the same as in Nereis, and that the aggregation in the drop, the appearance of the clear margin, and the growth of the aggregation are due to positive chemotaxis to CO_2.

This reaction is given clearly only by a fresh sperm suspension. One ten minutes old does not give it, owing presumably to formation of CO_2 in the suspension.

A considerable number of tests were made. In some the reaction was much more rapid than in the experiment described. In one of these tests I injected drops of 20 per cent, 4 per cent and 1 per cent of the CO_2 sea-water near together. In the case of the 20 per cent a ring with external clear margin was formed in a few seconds. The ring did not close. The 4 per cent formed a ring which closed in its center. There was no reaction to the 1 per cent. The same suspension gave no reaction twenty-five minutes after it was mixed. In another case I got a faint reaction to 1 per cent CO_2 sea-water.

The fact of positive chemotaxis of Arbacia sperm to CO_2 dissolved in the sea-water was repeatedly demonstrated. In the case of a drop mounted beneath a raised cover it expresses itself by a gradual aggregation of the sperm towards the center, leaving the margins clear.

As is to be expected from the slower and less delicate reaction to CO_2, as compared with Nereis, spermatozoa of Arbacia react also to other acids, but more slowly and not to so great dilutions. Thus in tests of N/10, N/50, N/250 and N/1000 H_2SO_4, strong positive reactions were obtained for the first three, whereas only a faint shadowy reaction is given to N/1000. In the case of Nereis

it will be recalled that N/2000 gives a distinct reaction. Positive reactions were not secured with N/1000 HCl or HNO_3, but were with N/250. The ring formed in response to N/250 H_2SO_4 grows somewhat owing to diffusion of the acid; in the case of N/50 and N/10 H_2SO_4 the growth is much greater, in general in proportion to concentrations.

As in Nereis alkalis agglutinate, but cause no aggregation. KOH was tested in two ways: (1) addition of a small quantity of N/2000 KOH in sea-water to a vial of fresh sperm suspension caused macroscopic agglutinations which are irreversible. (2) a drop of N/2000 KOH injected into a suspension beneath a raised cover fills in a short time with sperms that agglutinate; but there is no chemotaxis, and in a short while the drop is left to one side by aggregation of the sperm away from it, owing to rise of CO_2 tension elsewhere.

Reaction to egg-secretions

As contrasted with the slowness of reaction of Arbacia spermatozoa to acids, the reaction to egg-secretions is instantaneous and clear cut. There is a most pronounced positive chemotaxis, as tested with drops even of very weak egg-extract injected into a sperm suspension mounted beneath a raised cover-slip; but this is always associated with agglutination, and is, therefore, best considered under that head.

IV. AGGLUTINATION PHENOMENA AND REACTIONS OF SPERMATOZOA TO EGG-SECRETIONS

1. INTRODUCTION

Following the demonstration of definite chemotactic behavior of spermatozoa of Nereis and Arbacia the question naturally arises what relation, if any, has this form of behavior to the union of egg and spermatozoon in fertilization. As is well known, the mere observation of the interaction of the sexual elements in fertilization led long ago to the theory that the egg attracts the spermatozoon to itself by chemotaxis, and fusion results from active penetration of the ovum by the spermatozoon. But in recent years there has been a tendency to deny both of these

principles as factors in the union. Chemotaxis has fallen into disrepute; and the theory that the spermatozoon bores into the egg has been rejected by several observers.

If chemotaxis is concerned in the union of ovum and spermatozoon the medium in which fertilization operates must contain the substance concerned. In the case of the eggs of Nereis and Arbacia, therefore, the hypothetical substance which attracts the spermatozoa must exist in sea-water which has been in contact with fertilizable eggs; and it must be possible to obtain a sufficient concentration of the substance in question in sea-water to demonstrate its presence by reaction of the spermatozoa, because, *ex hyp.*, the substance exists in effective amounts in the sea-water surrounding the eggs. If it were impossible to demonstrate the presence of an agent to which spermatozoa of the same species are positively chemotactic by such means the theory of chemotaxis would have to be abandoned. However, the presence of such a substance is readily demonstrated both in Nereis and Arbacia.

In the second place, if the union of the ovum and spermatozoon after they have come in contact operates not mechanically, but through some bio-chemical reaction between spermatozoon and ovum, the sea-water in which eggs have been standing should contain a substance also capable of reaction with the sperm, which should be an efficient indicator for it.

I was guided by some such ideas as these in the series of experiments which follow, and which showed at the very first trials that sea-water which has stood with fertilizable eggs of Nereis or Arbacia contains a substance to which the spermatozoa of the same species are positively chemotactic, and also a substance which agglutinates the spermatozoa of its own species. It may be that one substance is concerned in both reactions, but it is more probable that two are present. It is perhaps worth emphasizing here, for this is the fact that struck me at the start, that the sea-water which has stood over eggs[3] combines both the effects of

[3] To avoid the frequent repetition of such a circumlocution we may call sea-water, which has contained eggs and is charged with their emanation, egg sea-water; and the concentration of the substance in the sea-water may be expressed by writing the relative bulks of eggs and sea-water as a fraction. Thus 'egg sea-water 1/3' would indicate that the bulk of eggs was one-third the volume of the sea-water. Time is also a factor in the concentration, of course.

an acid (aggregation) and also an alkali (agglutination) on the spermatozoa. The comparison may, of course, be superficial, but it serves at least to emphasize the double action of the egg-secretion.

As contrasted with the difference in rapidity and delicacy of reaction between the spermatozoa of Nereis and those of Arbacia to inorganic substances, we may note in advance that the reactions to the egg extractives are as rapid and clear in the one case as in the other, and are entirely similar in principle, though there are certain secondary differences that will be noted in the proper place.

2. INITIAL EXPERIMENT

We may begin by describing the reactions to be observed in the case of an Arbacia sperm-suspension freshly made and mounted beneath a raised cover-slip, into which a drop of Arbacia egg sea-water 1/10 to 1/20 about half-an-hour-old is injected. The naked eye observation shows almost instaneous formation of a ring at the margin of the drop, with simultaneous formation of a clear external zone about 1.5 to 2 mm. wide; the ring then breaks up into small agglutinated masses and so becomes beaded. The trail of substance left in withdrawing the pipette extends to the margin of the cover-slip. It also is a center of attraction and the ring is therefore prolonged by a chain of agglutinated masses to the margin.

One can observe the details of the reaction best under the microscope, using a low power, by bringing the point of the pipette into the field of the microscope and blowing in the drop with the aid of a flexible rubber tube held in the mouth, while looking through the microscope. The reaction takes place so rapidly that it requires repeated observations to observe all the details. In the first second the spermatozoa are aroused to intense activity and form small agglutinated masses within the drop; these then appear actually to 'rush' together (to use the language of my note book) to form larger agglutinations for a period of three to five seconds, after which no more fusion of masses takes place. The agglutinated masses thus range from relatively large to relatively small. While this has been going on in the interior of the drop, a ring

has formed at the margin, and a clear zone arises external to it. The ring is at first continuous, but it ruptures in numerous places in two or three seconds and each segment contracts quickly to an agglutination mass.

The agglutinated masses in the interior of the drop are smaller than in the ring, owing to the relatively low concentration of the sperm suspension within the drop, and they break up very quickly while the sperm is still extremely active. The movements of the spermatozoa then gradually slacken; in a few minutes the larger and more firmly agglutinated masses of the ring also begin to break up and in ten or fifteen minutes all are resolved.

This preliminary observation demonstrates a three-fold action of the egg-extractive: (1) it activates the spermatozoa; (2) it aggregates them through positive chemotaxis; (3) it agglutinates them. The phase of increase of activity lasts only a short time, a minute or two at the most, after which movements of the sperm slacken and become less than the control, or cease entirely. Positive chemotaxis (aggregation) is shown by formation of a clear zone external to the marginal ring. This is always a sign of chemotaxis, as we have seen in the preceding section.

The agglutination phenomenon is fundamentally different from the aggregation; in the latter the spermatozoa are merely loosely associated, and slight agitation is sufficient to scatter them. In the agglutinated masses the spermatozoa are stuck together and are not separated by shaking. In the case of Nereis where the agglutination is firmer than in Arbacia the masses may be broken up into smaller masses by needles, or preserved *en masse* in killing fluids. The breaking up of the ring into separate masses is a characteristic agglutination effect; the rings formed in response to an acid do not break up unless the acid is very weak (see p. 537). Finally an agglutinative substance produces its effect when shaken up and evenly distributed in a vial of sperm suspension, but an aggregative substance cannot of course exert a chemotactic effect in the absence of a gradient.

The same experiment succeeds well with Nereis. The eggs of this form give off a substance (or substances) into the sea-water, which causes aggregation and agglutination of the spermatozoa

when a drop of sea-water so charged is injected into a fresh sperm suspension beneath a raised cover slip. The activation is not so pronounced in this case as in Arbacia. The aggregation phenomenon is the same. The agglutinations are substantially permanent in Nereis; the spermatozoa stick together much more firmly.

In what follows we may leave the activation and aggregation phenomena out of account for the most part and confine ourselves to the problems of agglutination. The substance which causes agglutination of the spermatozoa we shall call the sperm agglutinin. The agglutination may be shown very strikingly in a vial of fresh sperm suspension. In the case of Arbacia the addition of two or three drops of egg sea-water 1/4, which has stood half-an-hour, to about 2 cc. of a fresh milky sperm suspension causes formation of agglutinations 1 to 2 mm. in diameter in a few seconds. The agglutination may be so strong that the fluid between the white agglutinated masses appears perfectly clear. The masses gradually fade from view in a few minutes, but microscopic agglutinations may remain half-an-hour or more.

The degree of agglutination is of course dependent on the density of the suspension. This is shown by the following experiment: Ovaries and eggs of Arbacia were cut up in about four times their own bulk of sea-water and allowed to settle. Two cubic centimeters of the supernatant fluid was put in each of three vials. To one was added 3 drops of a fresh milky sperm suspension, to the next 12 drops of the same, to the third 36 drops. No visible agglutinations formed in the first; in the second agglutinations became visible to the naked eye almost immediately, in the third agglutinations were larger, more numerous, and apparently more solid.

3. OVA ALONE PRODUCE THE AGGLUTINATING SUBSTANCE

The eggs of both forms thus produce an agglutinin in the sea-water. The next question is whether the agglutinin is specifically an egg-product. A considerable number of experiments prove that this is the case. The large body cavity of Arbaciais filled with abundant coelomic fluid and this may be supposed to

contain substances from various tissues. But it invariably proved perfectly neutral to spermatozoa of Arbacia, even when taken from females with large ovaries, showing that the substance concerned in agglutination does not escape from the ovaries of the intact animal, or, if its does, that it is promptly destroyed. Nor was it possible to extract a sperm agglutinating substance for Arbacia by extracting the intestine in sea- or fresh-water.[4] These experiments were repeated a sufficient number of times to be conclusive.

As illustrations: (1) Intestine extractives. August 31, 1912. The intestines of several Arbacia were cut up in about twice their bulk of distilled water, and were allowed to stand in it about an hour. The strongly amber-colored fluid was filtered off and rendered isotonic with the sea-water by addition of concentrated sea-water (four parts of the latter to six of the intestine extract). This fluid causes no agglutination in sperm suspensions. A similar extract of the ovaries caused immediate large dense agglutination masses. (2) Coelomic fluid: As is well known, the coelomic fluid of Arbacia contains large numbers of densely pigmented corpuscles. Outside the body the fluid quickly forms a loose clot which includes many of the corpuscles. The others can be separated from the remaining serum by centrifuging. The corpuscle-free serum was sometimes used, sometimes simply the clot-free serum. Repeated tests were made both by injecting drops into fresh sperm suspensions beneath raised cover glasses, and also by mixing with fresh sperm suspension in vials. Whether the coelomic fluid came from males or females it proved invariably negative, except for the faintest sort of agglutination reaction in one or two cases only, which may have indicated some individual differences. It is interesting in view of these facts that outside of the body the coelomic fluid becomes heavily charged with the sperm agglutinin if eggs are placed in it. It must be supposed therefore that the ovarian membrane is impermeable to the agglutinating substance in the intact animal.

[4] The experiments this year simply opened up the problems, and it was impossible to make any quantitative tests or adequate chemical examination. For the purpose of the biological problem of the behavior of the spermatozoa with reference to the eggs the question of immediate importance was the behavior with reference to egg-extractives or secretions in the sea-water tested. Stronger agglutinating solutions were made by increasing the quantity of eggs with reference to the sea-water, or by crushing the eggs in sea-water. Distilled water was shown to extract more agglutinin from a given bulk of eggs than sea-water; and the coelomic fluid of Arbacia also proved to be a better medium for extracting agglutinin from the eggs than sea-water. It would be possible of course to establish quantitative values for all of these relations, and this problem should receive attention. The problems of solubility of the agglutinin in various media, and other chemical questions, also present themselves.

In the case of Nereis it was not possible to reach such conclusive results, because the sexually mature female is practically a bag of eggs, and one cannot obtain other organs for testing. I cut up five females that had shed their eggs in 10 cc. of sea-water. In spite of efforts to get rid of all eggs, a considerable number were in the water. For control I used the eggs of two females in 100 cc. sea-water. On test in half-an-hour the fluid from above the eggs was found to be about ten times as agglutinative as the fluid from the bodies of the spent females. So that it is certain that other tissues do not produce much sperm agglutinin and it is probable that they do not produce any. The small amount present could be accounted for by the few eggs included, and perhaps by egg secretions absorbed by the tissues.

4. FIXATION OF THE AGGLUTININ BY SPERMATOZOA

The next question was whether the agglutination reaction as described has the usual characters of a chemical reaction? The general result is (1) that an agglutinated sperm suspension in which reversal has occurred is not capable of re-agglutination by addition of more of the agglutinating substance and (2) that the agglutinating substance disappears from an agglutinated suspension if not present originally in excess.

As regards the first point, the earlier experiments were concerned entirely with the form and conditions of the reaction, and the agglutinating substance was always used, as later results showed, in excess. It was not possible to get a repetition of the agglutination reaction under these circumstances. But one can get a repetition of the reaction in a sperm suspension by addition of successive small amounts of the agglutinating substance, until the reaction is complete, as the following experiment shows:

September 4, 1912. *Arbacia.* 2 cc. of a creamy active sperm suspension was agglutinated with 5 drops of an egg-extract prepared as follows: The ovaries of three females were cut up in about three times their volume of distilled water and allowed to stand about thirty minutes. Then the water was filtered off and made isotonic with sea-water by the addition of concentrated sea-water (proportions of 58 to 42 parts). This made a very strong agglutinating extract. After reversal of the agglutination described above the agglutination was repeated by addition of a drop of

another egg-extract. This time the agglutination was complete for it could not be repeated a third time. Other sperm suspensions gave similar results.

The result might be interpreted as a purely biological reaction, that is to say in terms of stimulation, were it not for the fact that the agglutinating substance disappears from an agglutinated sperm suspension, as shown by the following experiment:

September 12, 1912. *Arbacia.* Nine parts of a thick active sperm suspension was agglutinated by one part egg-extract. The agglutination produced was so strong that the fluid between the white masses appeared clear to the eye. In three or four minutes reversal of the agglutinations had begun. The agglutinated sperm suspension was then centrifuged until practically all the sperm was precipitated. The supernatant fluid was tested and agglutinin was shown to be absent. As control, a dilution of one part of the same egg-extract with 9 parts of sea-water was tested with the same sperm and proved to be strongly agglutinative. Three tests were made with each with uniform results.

There can be no doubt, as the result of this and other observations also, that the spermatozoa fix in some way the agglutinating substance, and it will be simplest to assume as a working hypothesis that the fixation is due to chemical union. I have not yet had the opportunity to ascertain if the agglutinin could be regained from the sperm precipitated in the centrifuge.

5. NATURE OF THE EFFECT ON THE SPERM

We have noted four effects of the egg-extracts on sperm of the same species, namely: (1) Stimulation of intense activity, which is of brief duration. This is more marked in the case of the sperm of Arbacia, than in the case of the naturally extremely active sperm of Nereis; (2) An orienting effect expressed in positive chemotaxis; (3) An agglutinating action; (4) Following these effects more or less complete paralysis of the sperm.

To what kind of change in the individual spermatozoa is the agglutination reaction due? We may note in the first place that the agglutination is between the heads of the spermatozoa, and that the tails are apparently unaffected, at least at first; it is only in later stages of the action of the agglutinating substance that the locomotor function is injured. The adhesion of the heads

demonstrates some change in the membrane that renders them sticky. The cells are so minute that it is difficult to observe any microscopical change in the case of Arbacia; in Nereis the spermatozoa are larger, and it can be seen that in agglutinated masses the heads of many of the spermatozoa are swollen into spherical form and have lost the normal strong refringibility. The change is in this case a very characteristic one, indicating a great increase in permeability. The spermatozoa which have undergone this change are usually motionless, and, when not fused with one another, appear to be glued to the slide or cover slip, never freely suspended.

Agglutination in itself is in no sense a specific reaction, but one that may be expected to accompany certain superficial changes of the spermatozoa, however caused, under conditions that bring the spermatozoa into contact. It occurs, to a limited extent, spontaneously in sperm suspensions that have stood for some time. It is particularly noticeable in Nereis under the following conditions: A fresh sperm suspension is allowed to aggregate on a slide beneath a raised cover slip and the aggregations remain undisturbed. In the course of ten or fifteen minutes small agglutinated masses may form around the margins of the aggregations or beneath the aggregations in contact with the slide. There may be twenty to fifty or more such masses associated with a single aggregation, and they are quite similar in their general appearance, to those produced suddenly by the agglutinin of the egg, though much smaller, on the average.

It is not probable that the agglutination is in any real sense toxic or cytolytic. It is true that the agglutinin inhibits movement after a few minutes, and it certainly lessens the fertilizing power of the sperm. But if an agglutinated mass of spermatozoa of Nereis be crushed under the microscope many of those liberated are active. Moreover, if a small quantity of an agglutinated suspension of spermatozoa be added to a relatively large quantity of sea-water fertilizing power is partially regained. This might be either because some of the spermatozoa of the agglutinated suspension had escaped combination with the agglutinin and were alone concerned in the actual fertilization, or because of recovery

from the agglutination effect. It is very difficult to form a definite opinion as to the real nature of the agglutination effect.

6. THERMO-RESISTANCE OF THE AGGLUTININ

The agglutinating agent is slowly destroyed at 95°C.:

August 28. *Arbacia*: (1) Ovaries and eggs of Arbacia were cut up in three times their bulk of sea-water, and let stand about an hour. The supernatant fluid is strongly agglutinative on Arbacia sperm suspensions. (a) Part of it was now taken and boiled about thirty seconds, and cooled. On test it proved as agglutinative as before. (b) Some more was then boiled five minutes and cooled. Its agglutinative power was apparently undiminished. (c) Another egg-extract similar to the first was then boiled and put in a beaker of boiling water for thirty minutes; the temperature stood about 95° during this process. On test its agglutinative power was shown to be greatly diminished. (d) In a fourth test some egg-extract was kept at 95° for sixty-six minutes. It still exhibited some agglutinative power, which, however, was very slight as compared with the control. The heated egg-extract exhibited a considerable change of color from the yellowish red of the control to a much brighter red.

Simultaneously with the loss in agglutinating power, it appeared also to gain in aggregating power: Drops of the heated and the unheated egg-extract were injected into the same sperm extract beneath a raised cover. The drop of the heated expanded its sphere of influence shown by immigration of the sperm about twice as rapidly as the unheated; this was tested several times; so that it would appear that the aggregative and agglutinative agents are probably distinct, and that the agglutinin inhibits aggregation to a considerable extent.

Nereis: The eggs of three females were inseminated in 9 cc. sea-water; the supernatant fluid has a slight amberish-green color, and is strongly agglutinative on Nereis sperm. (a) After keeping at 95° C. for ten minutes its agglutinative power was much reduced. (b) After twenty-two minutes at 95°C. the agglutinin was entirely destroyed. The color was entirely destroyed also.

Thus the agglutinating substances, whatever they may be, are either volatile being gradually driven off by heating, or they are slowly coagulated or disintegrated chemically by a temperature of 95°C. The agglutinin of Nereis is either more volatile or more labile than that of Arbacia. It is impossible to say definitely to what class of chemical substances these agglutinating

substances belong. It is, however, extremely improbable that they possess a degree of chemical simplicity sufficient to allow of volatilizing; it is more probable that they undergo slow chemical disintegration at the temperature employed. The thermostability of these sperm isoagglutinins is relatively very high, and this perhaps makes it doubtful whether they can belong to the same class of substances as the haem-agglutinins of vertebrate blood sera. This matter must therefore remain undecided, and it should be understood that the term agglutinin is used in the present paper in a purely descriptive sense.[5]

7. FERTILIZING POWER OF AGGLUTINATED SPERM

The powerful effect of the egg-extract on spermatozoa of the same species may be shown by a complete loss of motility as we have already seen, and also by a corresponding loss or diminution of the fertilizing power. The following experiments illustrate this:

1. Arbacia. The egg-extract used was made by cutting up the ripe ovaries in about three times their bulk of distilled water; in half-an-hour the water was filtered off and was then made isotonic with sea-water by the addition of 42 parts of condensed sea-water to 58 of the egg-extract. Five small watch crystals in a series contained (1) 8 drops of the egg-extract (2) 4 drops egg-extract, + 4 drops sea-water (3) 2 drops egg-extract, + 6 drops sea-water, (4) 1 drop egg-extract, + 7 drops sea-water (5) 8 drops sea-water. To each of these 3 drops of opalescent sperm suspension was added; and after twelve minutes a drop of a suspension of fresh eggs was added to each. We thus had the same quantity of eggs in sperm suspensions of the same density, but in graded amounts of egg-extract. The sperm suspensions were so dense that in the control (no. 5) the jelly became packed with sperm, forming dense halos around the eggs. In (4) a very few (about 1 per cent) had slight halos of sperm in the outer layer of the jelly, but in (1), (2) and (3) the paralysis of the sperm was so complete that they did not enter the jelly of the eggs at all. Ninety-seven minutes later none of the eggs in (1), (2), (3) or (4) had segmented; whereas at least 5 per cent of the control were now in the two-celled stage. The lot of eggs was rather poor in this case, but fertilization was confined entirely to the control.

If the experiment be made in another way some recovery of the spermatozoa from their state of paralysis may be observed. Thus: An

[5] It should be borne in mind that but little is known concerning lysins or agglutinins of invertebrates. It is perhaps not to be expected that they should exhibit the same degree of thermolability as those of vertebrates.

active sperm suspension was divided in two parts, and one part was agglutinated by the addition of about 40 per cent of its own volume of the egg-extract described above, to the other an equal amount of sea-water was added. The first was strongly agglutinated; after reversal both suspensions were stirred up, and beginning thirteen minutes after agglutination a series of fertilizations were carried out by adding one drop of the agglutinated sperm suspension to a measured quantity of eggs in about 9 cc. of sea-water at 10 minute intervals. Each fertilization had a control of the same quantity of eggs fertilized with one drop of the control sperm. The consistent result was that about 16 per cent of the eggs fertilized with the agglutinated sperm segmented and at least 33 per cent of the control. The non-agglutinated spermatozoa are about twice as effective as the agglutinated. But a considerable degree of recovery of some spermatozoa of the agglutinated suspension is shown.

2. Nereis. Experiments with Nereis did not give such a marked reduction of the fertilizing power of agglutinated sperm as in Arbacia. There was, however, a marked delay in the formation of jelly when agglutinated sperm was used as compared with normal sperm.

It is somewhat difficult to make a satisfactory interpretation of the effect of agglutination on fertilizing power. On the one hand we may suppose that a certain proportion of spermatozoa resist the agglutination effect, and are alone concerned in any fertilizing power of an agglutinated suspension; on the other hand it might be supposed that the agglutinating effect does not modify fertilizing power except as it decreases the motility of the spermatozoa or that the effect is reversible under the condition of the experiments. Either assumption would be consistent with the facts.

8. CONDITIONS OF FORMATION OF THE AGGLUTININ BY THE EGGS

The conditions of excretion of the agglutinating substance by the eggs into the sea-water is quite different in the two forms. In Arbacia the agglutinin is excreted continuously by unfertilized eggs in such amounts that I have not succeeded even by repeated washings in removing it all. Fertilization does not appear to increase or decrease the quantity. In Nereis, on the other hand, unfertilized eggs secrete but little of it, and one or two washings in sea-water will completely remove it so that the eggs secrete no more in detectable quantities; but at the moment of fertilization, on the other hand, it is poured forth in advance of the jelly in

large quantities and the eggs then appear to have disposed of their entire store, for washed fertilized eggs no longer produce it. The conditions are important in their bearing upon the question of permeability of the egg-membrane with reference to fertilization. The spermatozoa are very efficient indicators of substances leaving the egg. In the case of Nereis it can be shown that there is a sudden increase of permeability at the moment of fertilization, but in Arbacia such evidence is lacking.

The conditions in Arbacia may be shown by the following experiment: Eggs were washed free from all fragments of ovary and placed in about 20 times their own bulk of sea-water and divided in three lots, *a*, *b* and *c*. The sea-water over them agglutinates Arbacia sperm instantaneously. This test was made at 9.30 A.M. The supernatant fluid was then removed and the eggs washed in 20 times their bulk of sea-water. At 9.53 the supernatant water was tested and found agglutinative. 9.58, *a* and *b* were again washed. 10.04, supernatant fluid again agglutinative. 10.05, lot *a* was fertilized with 2 drops of sperm. 10.10, *a* and *b* tested again; both agglutinate. 10.16, *a* and *b* washed again. 10.23, supernatant fluid of both agglutinates. 10.30, *a* and *b* washed again. 10.36, both agglutinate sperm; *b* is rather more effective. 10.40, washed *a* and *b* again. 10.45, both agglutinate; *b* more effective. 10.51, washed *a* and *b* again. 11.00, both agglutinative; *a* more than *b*. 11.08 to 11.20, other tests of *a* and *b* show *a* somewhat more effective.

The experiment shows both fertilized and unfertilized eggs of Arbacia to be constantly secreting a substance into the sea-water which agglutinates the spermatozoa. The substance must be effective in very minute quantities for the amount of water used in each of the eight washings was at least ten times the bulk of the eggs, yet as soon as the eggs had settled to the bottom of the vials used the supernatant fluid contained the agglutinin in appreciable amounts. How long this process keeps up in Arbacia I cannot say; and too much reliance cannot be placed on the result for the fertilized eggs because a large proportion of the eggs failed to fertilize or at least to segment.

The conditions in Nereis are quite different; the experiments showed that shortly after the eggs are taken they charge at least ten times their bulk of sea-water with an easily detectable amount of sperm agglutinin. But if the eggs are washed once usually no more agglutinin can be demonstrated. If now the eggs are stirred

up in the vial, fertilized and allowed to settle, the supernatant fluid is very powerfully agglutinative. The experiment was repeated a sufficient number of times to make certain of this result.

A number of tests were also made with the object of determining if the eggs of Nereis continued to produce agglutinin after fertilization. These showed that the eggs cease very quickly their production of agglutinating substance. None could be detected during the maturation period, but apparently there is a second production about the time of the first cleavage. On this point I wish to be understood to speak with reserve. The swelling of the jelly secreted at fertilization makes the eggs very bulky, and the jelly itself takes up any egg secretion, so that there are considerable technical difficulties in making satisfactory tests.

The fact that stands out perfectly plainly in the case of Nereis is the sudden increase in secretion of agglutinin into the seawater just after insemination, followed by cessation of its production. The spermatozoa give absolutely positive tests. As will be shown later, the observations on the normal fertilization are in complete harmony with this.

The difference between Nereis and Arbacia in these respects is thus sharply marked. However, it should be remembered that the unfertilized eggs of Arbacia have formed both polar bodies, whereas those of Nereis are in the stage of the germinal vesicle. It may be that eggs of Arbacia in the germinal vesicle stage are relatively impermeable in the same sense as those of Nereis.

9. HETERO-AGGLUTINATION AND THE QUESTION OF SPECIFICITY: REACTIONS BETWEEN NEREIS AND ARBACIA

The demonstration of intraspecific sperm-agglutinating substances derived only from the ova having been made, the question arose whether these substances were essentially the same in both species, or different. If the same, the egg-extract of each should agglutinate the sperm of the other. A number of tests were therefore made which demonstrated conclusively that the substances are decidedly different with reference to their cross-agglu-

tinating effects, and this result has raised a number of questions, the most important of which relate to the question of specificity, but which could only be defined in the time at my disposal and not definitely answered.

The first suggestion that the sperm agglutinating substances of Arbacia and Nereis are different came from the following experiment: A raised coverslip preparation of Nereis sperm was made in the usual way and into it were injected a drop each of (1) Arbacia egg-extract in sea-water, (2) drop a of coelomic fluid from a female Arbacia, (3) a drop of coelomic fluid from a male Arbacia. All three caused very extensive firm agglutinations of the Nereis sperm.

Thus the egg-extract of Arbacia contains an agglutinating substance for the Nereis spermatozoa as well as for its own; but the coelomic fluid of Arbacia also causes agglutination of the Nereis spermatozoa, whereas it is perfectly neutral with respect to its own. This demonstrated, therefore, the existence of at least two sperm-agglutinating substances in Arbacia, namely: One in the egg-extract agglutinative for its own sperm and that of Nereis, and one in the coelomic fluid not agglutinative for its own sperm, but agglutinative for the foreign sperm of Nereis. The probability is, therefore, that the egg-extract contains both substances seeing that it is agglutinative for both kinds of spermatozoa. This was afterwards demonstrated.

The reciprocal experiment proved the difference of the Nereis and Arbacia agglutinating substances conclusively, for it was shown that Nereis egg-extracts strongly agglutinative for sperm of Nereis had no agglutinating effect on Arbacia sperm within the limits of attainable concentrations (several experiments). The Arbacia fluids are extremely toxic apparently to the Nereis sperm for the agglutinations were more solid than those caused by the iso-agglutinating substance. The absence of a reciprocal effect, that is, of Nereis extracts on Arbacia sperm, is therefore all the more striking.

The same difference may be shown by the reactions of sperm suspensions of both species to one another. The experiment was made in the following manner:

July 28, 1912. *a*. A suspension of active Arbacia sperm was made at 11.23 A.M.

b. A suspension of active Nereis sperm was made at 11.24 1/2.

The two suspensions were made of equal density as far as possible.

Part of each suspension was then mounted on a slide beneath a raised cover-slip.

A drop of the Nereis sperm was then injected into the Arbacia slide (slide 1) and a drop of Arbacia sperm into the Nereis slide (slide 2).

Slide 1 gave a very faint reaction; only slight evidence of a ring formation at the margin of the Nereis drop.

Slide 2, on the other hand, gave a very pronounced reaction due to inwandering of the Nereis sperm into the Arbacia drop, followed by agglutination of the inwandering sperm.

The difference in reaction is due to two circumstances: (1) The Nereis sperm exhibit a more pronounced chemotaxis than the Arbacia sperm, hence they tend to enter the drop of Arbacia sperm, whereas on the other slide the Nereis sperm tend to diffuse from the drop into the Arbacia suspension. (2) The Nereis sperm that wander into the drop of Arbacia sperm are agglutinated, but there is no reciprocal reaction of the Nereis sperm on any inwandering Arbacia spermatozoa.

It is demonstrated, therefore, first that Arbacia fluids in general are toxic for Nereis spermatozoa to the extent, at least, that they cause agglutination; but on the other hand, that no secretion of Nereis appears to cause agglutination of Arbacia spermatozoa; and second that the eggs of each species produce an agglutinin for the sperm of its own species.

Agglutination is not in itself a specific process; it may take place spontaneously to a certain extent under some conditions; it is caused by increase of alkalinity of the sea-water in the case of Nereis and Arbacia, or by the action of certain foreign sera as in the case of the action of Arbacia fluids on Nereis sperm. On the other hand, the class of specific immune agglutinins, characterized by limitation of their action to the specific form of blood or sperm used as antigen is well known. The question naturally arises, therefore, to which class the iso-agglutinating substances produced by ova of Arbacia and Nereis belong.[6]

[6] In the latter case, fertilization itself would have to be regarded as an immunizing process, the sperm acting as antigen after entrance into the egg. It seems, in

The fact that the egg secretions of Arbacia cause agglutination of Nereis sperm as well as Arbacia sperm seems at first sight to indicate the iso-agglutinating substance of Arbacia is not specific.* But the fact that other Arbacia fluids likewise agglutinate the sperm of Nereis, but not those of Arbacia, raises the question whether the egg-secretion does not contain in addition to the iso-agglutinating substance, also another which is agglutinative for Nereis sperm like the substance in the coelomic fluid, but not for its own sperm.

If there are two substances present in the egg secretion it ought to be possible to separate them by various means. They might exhibit different heat-lability, so that one might be destroyed at a temperature that would leave the other still active. Of if they have different affinities it might be possible to fix the Nereis agglutinating substance by Nereis sperm, leaving the iso-agglutinating substance intact. Neither of these experiments could be performed this year, owing to the disappearance of the necessary material.

However, it was possible to show in another way that the Nereis-agglutinating substance of Arbacia egg-extract is distinct from the iso-agglutinating substance: An egg-extract of Arbacia seventeen days old was found to have entirely lost its power of agglutinating Nereis spermatozoa, while it retained undiminished its power of agglutinating Arbacia spermatozoa. Originally it agglutinated both kinds of spermatozoa. Now the change that took place in the egg-extract on standing is not a mere weakening of action as might be supposed, because the iso-agglutinating action was noted as undiminished, whereas the hetero-agglutinating action was entirely lost. The only possible conclusion, therefore, is that the egg-extract contained two agglutinating substances at least, namely: An iso-agglutinin and a hetero-agglutinin, and that the latter is relatively labile, the former relatively stable. Unfortunatelly the experiment could not be repeated on account of the total disappearance of the material.

fact, an almost necessary conception on the general principles of immunity phenomena that the sperm should so act. The question would be, of course, whether there is a connection between any antibodies so formed and the sperm iso-agglutinins produced by the next generation of ova.

The experiment in detail was as follows:

September 15, 1912. One small male Nereis available. Its sperm is aggregative and very active. The Arbacia egg-extract was of August 29 and was made by cutting up ovaries and eggs of Arbacia in four times their volume of sea-water. After settling of the eggs the supernatant fluid was poured off, and had been kept in a stoppered vial since.

1. A drop of the Nereis sperm and a drop of the egg-extract were placed side by side on a slide and connected; the sperm diffused into the egg-extract and swam around in it; no agglutination.

2. A raised cover mount was made of the Nereis sperm and a drop of the egg-extract injected. The sperm entering the drop swam around, and were not agglutinated.

Controls: 1. The sperm of Nereis was agglutinated immediately by extract of Nereis eggs kept since September 8.

2. The egg-extract of Arbacia agglutinated Arbacia spermatozoa with no apparent diminution in the strength of the reaction.

Conclusion: The nonspecific agglutinating substance has been destroyed by the chemical changes in the extract in the course of seventeen days; but the iso-agglutinating sperm substance still remains.

This experiment does not demonstrate that the sperm iso-agglutinin of Arbacia egg-extract is specific, but merely that it is without effect on the Nereis sperm, just as the iso-agglutinin of Nereis eggs is without effect on Arbacia sperm. It is of course still possible that the iso-agglutinating substances might have an agglutinating effect on some other varieties of sperm, and a crucial test of specificity must await the securing of new material.

However, it seems to me that the probabilities in the case lie strongly on the side of specificity of these sperm iso-agglutinating substances. Quite apart from the value of the evidence already adduced, we must consider the general fact that ova and spermatozoa of the same species do behave in a specific way with reference to one another in the process of fertilization. This must have some chemical basis and on the chemical side the only reactions that exhibit a corresponding degree of specificity are those between antigens and anti-bodies in the field of immunity. We have two parallel instances, therefore, and the slight evidence which I have so far been able to bring forward in favor of the specificity of the sperm iso-agglutinins of ova gains immensely in weight by its association with the universal principle of specificity in fertilization, and the known class of specificities in agglutination reactions.

The agglutination of spermatozoa is, of course, in itself of no significance for the problem of fertilization; the spermatozoa unite in fertilization with the egg, not with one another. The agglutination reaction is, however, an indicator of an important change in the spermatozoon in the presence of egg secretions, and therefore evidence of a change that any spermatozoon must undergo when it comes in contact with the egg. The adhesive property that the sperm develops under these circumstances may be an important factor in binding the sperm to the egg until it can be incorporated. But, if the reaction be specific, it is much more than this; it is evidence of an intimate chemical combination of sperm and egg constituents which begins at the very moment of union.

Von Dungern's experiments ('02) are the only ones, so far as I know, in which the production of sperm agglutinins by ova was investigated, and he discovered only hetero-agglutinins, no iso-agglutinins. He did, indeed, describe the loss of motility of spermatozoa in egg-extracts of the same species, but he entirely missed the phenomenon of agglutination and its reversal. He reveals the reason for this failure by his remark that he always examined for the effect of the 'egg-poison' about half-an-hour after its addition to the sperm; but the phenomenon of agglutination and its reversal are completed in about five minutes.

Von Dungern also made experiments on the production of immune sera by injection of ova and spermatozoa separately into rabbits, and found that both caused the production of a sperm agglutinin in the rabbit's serum. From this he concludes that both kinds of reproductive elements possess chemically identical complexes of molecules in the protoplasm. While this may be admitted as at least a very probable conclusion, his farther conclusion that fertilization does not depend upon any specific antagonism between ovum and sperm, but is conditioned by the similarity of their protoplasms, is not well founded, for the egg is a very complicated chemical system, and it certainly contains molecules antagonistic to sperm, even if, as Von Dungern's experiments indicate, it also contains some that are not.

10. INTERPRETATION OF SOME PHENOMENA OF NORMAL FERTILIZATION IN NEREIS

To observe all the details of normal fertilization it is desirable to inseminate in a suspension of India ink which will define the transparent substances exuding from the egg on insemination. the following observations can then readily be made on mixing a drop of the eggs in the ink suspension with a drop of opalescent sperm suspension: Hundreds of spermatozoa become attached to each egg almost immediately; those in contact with the egg do not show much activity, but are usually definitely oriented radially; the spermatozoa external to these are in active movement. In about a minute a clear fluid begins to exude from the egg and surrounds all attached spermatozoa and involves the immediate neighbors. The first exudate is quite fluid for it flows around the spermatozoa and does not sweep them away, but the movements of all the spermatozoa within the exudate cease suddenly. The flow continues, and then most of the spermatozoa are swept away from contact with the egg, for the later exudate is gelatinous in consistency. However, a good many spermatozoa remain in contact with the egg for some time, but these are detached one by one as the flow of the jelly continues, until only one remains. Some of the supernumerary spermatozoa are not carried away until five or more minutes after insemination.

The immediate prevention of polyspermy in Nereis appears to be due to the paralyzing effect of the egg-exudate poured out in response to the stimulus of the first effective spermatozoon. Polyspermy could take place in Nereis only under two conditions, namely, (1) if two or more spermatozoa *simultaneously* give the stimulus to the egg that causes excretion of the agglutinin; for the condition of stimulus appears to be that the spermatozoon be securely anchored to the egg; and all spermatozoa not securely attached at the moment the egg begins to secrete are prevented from securing attachment by the resulting paralysis; (2) if the reaction of the egg be slow and therefore localized at first to the region of an effective spermatozoon, opportunity will be afforded for attachment of other spermatozoa.

In a short time the egg develops a physiological condition in which union of spermatozoa is no longer possible. The immediate protection against supernumerary spermatozoa is, however, afforded by the paralyzing action of the egg-secretion.

Union of ovum and sperm, prevention of polyspermy, and the attainment of a condition of insusceptibility to other spermatozoa are phenomena so closely related in time sequence that a casual connection must be postulated. These can be brought under one head, in the case of Nereis at least, if we assume that the substance that paralyzes all the sperm in the vicinity of the egg is necessary for the actual fusion of the spermatozoon and egg and is completely used up in the cortical changes that follow immediately on insemination: the condition of insusceptibility would be due to loss of a necessary substance, the immediate prevention of polyspermy to paralysis of all ineffective spermatozoa, and the penetration of the sperm to a chemical change of the effective sperm and the neighboring egg-cytoplasm involving physical alterations in surface tension, viscosity, et cetera.

In the case of Arbacia, I have been unable to demonstrate an increase of secretion from the egg into the sea-water at the moment of insemination nor yet cessation of such secretion soon after insemination; however, as indicated before (p. 560), this failure may be due to the presence of unfertilized eggs in the experiments, which require to be repeated.

11. SUMMARY: PART IV

1. The ova of Nereis and Arbacia give off into the sea-water a substance (or substances) which agglutinates the sperm of their own species. The sea-water, which has the agglutinating substance in it, has also a substance to which the spermatozoa of the same species are positively chemotactic.

2. The eggs alone produce the sperm agglutinating substance; It cannot be extracted from other tissues.

3. The agglutinin disappears from a mixture of sperm suspension and agglutinin if not present in excess; the disappearance is attributed to chemical combination.

4. The agglutinating substances are highly thermostable, but are slowly destroyed by temperatures above 95°C.

5. In the presence of excess of the agglutinating substance spermatozoa of Arbacia lose their fertilizing power.

6. Eggs of Arbacia give off the agglutinating substance in the sea-water in large quantities prior to insemination; but eggs of Nereis give off only small quantities until inseminated, or until the cortical change analogous to membrane formation in the sea-urchin egg is somehow produced.

7. The egg-extract of Nereis does not agglutinate Arbacia spermatozoa.

8. The substance in the egg-extract of Arbacia that agglutinates Nereis spermatozoa is distinct from the iso-agglutinating substance.

9. The coelomic fluid of Arbacia contains a substance which agglutinates the spermatozoa of Nereis but not of Arbacia. Presumably this substance is the same as the hetero-agglutinating substance of Arbacia egg-extract.

10. Two arguments in favor of the specificity of the iso-agglutinative reaction were brought forward, namely, (a) The fact that the iso-agglutinin of Arbacia is distinct from the hetero-agglutinin in the case of Arbacia and Nereis; (b) that fertilization is fundamentally a specific reaction, and that the phenomena of agglutination belong in a class of phenomena in which specificity exists, and between elements which react specifically in fertilization. While admittedly not demonstrative these arguments appear to me to be cogent.

V. DISCUSSION

Since Pfeffer's fundamental investigations concerning chemotaxis of spermatozoa of ferns and mosses with reference to the secretion of the archegonia a similar explanation of the behavior of animal spermatozoa with reference to the eggs of the same species has been anticipated, and indeed has been postulated by many writers without any other experimental basis. However such actual experiments as have been performed have not been favorable to such an interpretation. Thus Buller ('00) experi-

mented with the spermatozoa of sea-urchins by the method of Pfeffer and came to the conclusion that "the spermatozoa of the Echinoidea are not attracted to the egg by means of any special substance excreted by the latter. The vast number of spermatozoa and the large size of the eggs are sufficient to ensure the necessary contact taking place." Von Dungern ('02) also rejects the idea of an egg-secretion attracting the spermatozoa in the case of sea-urchins and starfish. Morgan, Payne and Brown ('10) also accept Buller's interpretation, and there has recently been a tendency among biologists to reject chemotaxis of the spermatozoon as a factor in the fertilization of the egg.

Previous observers have worked either with Pfeffer's capillary tube method, or with the eggs themselves. I made a sufficient number of experiments with capillary tubes to convince myself that this method of experimentation is many times less effective than the method I employed. As illustration: July 4, 1912: I filled three pieces of capillary tubing with a concentrated solution of CO_2 in sea-water, with 10 per cent and 1 per cent of this solution and broke off short pieces—neither end of which had been in the solution—to be tested. These pieces were then introduced into an active sperm suspension of Nereis beneath a raised cover-slip. In the course of a few minutes a decided positive reaction was obtained with the first and second tubes, the sperm appeared to stream into the open mouth of the capillary tubes and soon formed white plugs at the mouths of the tubes. The tube containing 1 per cent CO_2 sea-water and a control tube with sea-water alone showed no reaction. In a repetition of this experiment the 1 per cent CO_2 sea-water and control were negative, and the 10 per cent showed only slight reaction. The diameter of the lumen of the tube was 0.48 mm. A much finer tube of 10 per cent CO_2 sea-water showed no reaction at all. With tubes of the size first used 10 per cent CO_2 sea-water is near the minimum for a positive reaction. But a drop of 0.5 per cent CO_2 sea-water injected into a similar suspension causes chemotactic response. Tubes of the size used are therefore about twenty times less effective as indicators of the chemotactic reaction, stated in terms of percentage of CO_2 required, than the injected drop method. It

is obvious that the size of the tube is a fundamental condition of the experiment, both because the diffusion is a factor of size and also because of the interference of thigmotactic reactions of the spermatozoa at the mouth of the tube with the purely chemotactic response. Buller does not state what was the size of the tubes that he used in his experiments. But, if the delicacy of the reaction was reduced to one-twentieth by the tube, his failure to get a positive reaction in tubes containing sea-water taken from over eggs is not surprizing.

Von Dungern drew his conclusions from observing the behavior of spermatozoa mixed with eggs. Many embryologists, like myself, have made hundreds of observations of this kind; but it is obvious that the conditions thus created render an analysis of the behavior of the spermatozoa impossible. In some experiments I introduced a drop of eggs in sea-water into a sperm suspension beneath a raised cover, and obtained the typical ring formation of spermatozoa with reference to the group of eggs considered as a whole. But within the group any evidence of chemotactic reaction is clearly impossible.

As to the rôle that chemotaxis as a principle may play in the fertilization of the ova in nature it is difficult to form a clear conception. It may be little and it may be considerable. In the first place it may be noted that, although the echinids have been favorite subjects for research, but little appears to be actually known concerning their breeding behavior. In the second place we do not know the distance to which the secretion from an isolated egg will diffuse. But even if we assume that it extends effectively only a short distance in terms of the egg-diameter the result would be essentially to immensely increase the chances of scattered spermatozoa to become entangled in the jelly of the egg. Measurements of the effective radius of diffusion of the egg-secretion could, I believe, readily be made by the method employed in my work and the results of this might enable us to form some clearer idea of the possible significance of chemotaxis taken by itself in the meeting of the germ-cells.

The present results merely show that it may be a factor of some significance. The quickness and readiness of the reaction of

spermatozoa of Arbacia and Nereis to the secretion of their own kind of eggs is certainly surprising.

Another effect of their secretions that should be taken into account in this connection is the stimulating effect on the spermatozoa. This is more marked in some animals than in others. Thus the spermatozoa of Nereis are so active in the sea-water alone that but little effect of the egg-secretions can be noted; in the case of Arbacia, although the sperm are quite active in pure sea-water yet the egg-secretions greatly increase their activity for a brief time. In the case of the star-fish, according to Von Dungern's account, the spermatozoa tend to be very inactive in pure sea-water, but are aroused to intense activity by the secretions of the ova.

In different animals, therefore, we may expect to find some difference in the effect of egg-secretions on the activity of the spermatozoa. But the fact that in such widely separated forms as Arbacia and Nereis secretions of the egg cause strong positive chemotaxis of the spermatozoa inclines one to the view that such a reaction may be very wide spread. In a form in which egg-secretions are both activating and directing in their action, the importance of such secretions in favoring the preliminary steps in fertilization can hardly be doubted.

The experiments, like Pfeffer's earlier ones, indicate that the factor of specificity is probably subordinate in the purely chemotactic response. CO_2 and acids are in no sense specific, but they are very effective chemotactic agents with Nereis spermatozoa. But the case of Arbacia serves to indicate that substances of the egg, whether specific or not, are more generally effective than simple chemical substances, for its requires such substances, apparently, in the case of Arbacia to produce a reaction of the spermatozoa comparable in quickness and precision to the reaction of Nereis spermatozoa to acid, CO_2, or the secretions of its own eggs.

We have seen that in some respects the chemotactic behavior of spermatozoa of Nereis and Arbacia is different depending on their relative sensitiveness to CO_2 and other agents. The much greater power of resistance of spermatozoa of Chaetopterus and

Loligo to CO_2 (see p. 528) indicates still greater differences in characteristic behavior. And it may be that the differences of chemotactic behavior of the spermatozoa of various animal phyla will turn out on investigation to be extensive. I am very far, therefore, from wishing to generalize any of the principles that we have found to hold true for Nereis and Arbacia. Sound generalizations must be based on much more extensive work. I have made some observations on the sperm of Platynereis megalops which demonstrate great differences as compared with Nereis in spite of the close relationship, correlated, no doubt, with differences in breeding behavior. While the common form of organization of flagellated spermatozoa points to fundamental principles of behavior in common, yet it must not be forgotten that each kind of spermatozoa has the chemical composition of the species, and may therefore have entirely specific forms of behavior.

The agglutination of the spermatozoa by normally formed egg-exudates of the same species indicates the possibility of studying the chemistry of fertilization directly through use of the spermatozoa as indicators. The very few and incomplete results which I was able to obtain in the time at my disposal seem to me to indicate a fruitful line of work. It would be interesting, for instance, to investigate whether or not the ova of hermaphrodite animals produce a sperm auto-agglutinin, that is, an agglutinating substance for spermatozoa of the same individual. Morgan's work on Ciona has shown that the failure to self-fertilize is in this case due to failure of penetration of the spermatozoon. It is difficult, as he points out, to explain this on any mechanical grounds. But if a specific agglutination is a necessary step in union of ovum and spermatozoon, the failure to produce an auto-agglutinin would explain the failure of self-fertilization. We would have in this event a precise parallel to the usual failure to produce blood auto-agglutinating substances in experiments on immunity, though iso-agglutinins are readily produced.

Godlewsky ('11) has shown that there is an antagonism between the sperm of certain animals (Chaetopterus and Echinids) which destroys the fertilizing power of each when mixed together for a certain length of time. He compares this to the antagonistic

action of heterogenous haemolytic sera on one another; and concludes that his results strongly confirm Loeb's theory that the spermatozoon initiates development by means of a lysin.

Without discussing the interpretation, and considering only Godlewsky's most interesting results, another parallel is furnished to immunity phenomena. We may confidently expect, therefore, that study of the reactions of spermatozoa will break a new path into the field of fertilization.

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STUDIES OF FERTILIZATION

VI. THE MECHANISM OF FERTILIZATION IN ARBACIA

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ONE FIGURE

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I. INTRODUCTION

The fifth study of this series on "The behavior of the spermatozoa of Nereis and Arbacia with special reference to egg extractives" forms the prelude to the present study. In that paper I dealt with the phenomena of activation and of aggregation (chemotaxis) of spermatozoa and with their agglutination by egg secretions of the same species. Regarding fertilization as involving chemical interaction of the ovum and the spermatozoon, I concluded from some of the modes of behavior of the spermatozoa, especially the agglutination phenomena, that "these small motile cells may prove better indicators of some of the reactions involved in fertilization than the slowly reacting egg" and that we might "confidently expect that study of the reactions of spermatozoa will break a new path into the field of fertilization."

The results described in the present paper have been obtained largely by the use of sperm-suspensions as indicators, but also by the study of an inhibitor of fertilization contained in the blood of certain individuals of the same species. These methods reveal a striking series of facts which appear to me to justify a somewhat new point of view in regard to some of the problems of fertilization. I have already briefly presented this point of view in a rather special form with an outline of the main facts in a preliminary paper (Lillie '13).

The essential conclusion is that fertilization is a reaction between three bodies of which one is born by the sperm and one by the egg; the third body, which is secreted by the egg, reacts with both the others. The spermatozoon functions essentially as an activator of the third body which I propose to name 'fertilizin;'[1] the latter when activated enters into certain reactions in the cortex of the egg which lead to membrane formation. In order to give a concrete working conception I have pictured the fertilizin as possessing two side chains active in fertilization, viz: one

[1] This is the same substance which I called the 'sperm isoagglutinin' in the preceding paper of this series, because it causes agglutination in sperm suspensions of the same species.

reacting with the sperm which I call the 'spermophile side-chain' or group and the other reacting with the egg which I call the 'ovophile side-chain' or group. The chemical group of the sperm which reacts with the fertilizin is named the sperm receptor and that of the egg the egg receptor.

We are thus furnished with a concrete conception which answers very well for purposes of description; it need not however, be taken too literally. The theoretical aspect of the results is discussed in section IV.

The present paper presents in some detail the evidences on which this general conception of fertilization rests; it deals exclusively with Arbacia; in a later paper I shall present some confirmatory evidence from the study of Nereis. We shall consider first the action of the spermophile side-chain of the fertilizin and the evidence that the latter is a necessary link in the fertilization process. Second, we shall consider the nature of the ovophile side-chain of the fertilizin, the means of inhibiting its action and of neutralizing such inhibition.

The terminology has been largely adopted from immunology, because it seemed best suited to express the facts. If it seems rather bizarre to the zoölogical reader I must ask him not to conceive prejudice for this reason against the facts themselves, which stand on their own feet quite apart from any terminology or theory. The terminology and the theory are significant only to the extent that they give a brief description of the facts, and serve as a working hypothesis.

II. THE SPERMOPHILE SIDE-CHAIN

I. A QUANTITATIVE METHOD OF STUDYING THE PRODUCTION OF FERTILIZIN BY OVA

The fundamental fact which forms our point of departure is that fertilizable eggs of Arbacia suspended in sea-water secrete a substance (fertilizin) for which only the spermatozoa of the same species constitute an efficient indicator. Similarly the only cells that produce the substance are the egg-cells of the same species; it is not found in the blood or in extracts of other tissues.

The presence of the substance is shown by the agglutination of sperm-suspensions of the same species. If a few drops of sea-water containing this substance in sufficient concentration be added to a 1 per cent sperm-suspension in a test-tube a flocculent condition becomes apparent to the naked eye in a few moments. The qualitative aspects of the reaction were described quite fully in the preceding paper of this series, and need not therefore be repeated here.

In my previous papers ('12 and '13) no attempt was made to establish a quantitative method of study. But in order to carry the subject farther it was necessary to determine how much of the sperm agglutinating substance was produced by the ova under various conditions. As noted in previous papers the reaction is reversible. With high concentrations of the agglutinating substance it may be several minutes before the agglutinated masses break up and reversal is complete; with low concentrations on the other hand the agglutinated masses are smaller and their disintegration is correspondingly more rapid. It was therefore possible to establish as a unit concentration of the agglutinating substance the greatest dilution at which an unmistakable reaction is given. Such a reaction lasts only a few seconds. It would be obviously very tedious to establish the *exact* point at which the reaction ceases in a long series of experiments, and in practice I prepared a series of dilutions each member of which was one-half of the preceding member, and the last one to give the reaction was regarded as the unit concentration. The method is as follows: Taking a given solution of the fertilizin a series of dilutions is made. If these begin with (a) 1/100 (i.e., 99 parts of sea-water plus 1 part of the solution) a half dilution of (a) is made, (b) (i.e., 1/200); a half dilution of (b) follows (c) 1/400, (d) 1/800, (e) 1/1600, (f) 1/3200, etc. If 1/1600 is negative, 1/800 may give a 4 to 6-second reaction; if 1/800 is negative 1/400 may give a 4 to 6-second reaction, etc; when the solution was known to be strong it was usual to begin with a 1/100 dilution; otherwise we might begin with a 1/10 dilution, followed by a 1/20, 1/40, etc., or 1/2 followed by 1/4, 1/8, 1/16, etc., the last member of a series to give a positive reaction was accepted as

of unit concentration. Thus any given solution could be rated as of 10, 100, or 6400, etc., agglutinating strength depending on the dilution test.

The most delicate method of determining the reaction is the raised cover-slip method described in a previous paper ('13). The sperm-suspension used as indicator should be about 1 per cent (i.e., one drop dry sperm to about 99 drops sea-water) and should be not more than 10 minutes old. A few drops of this are mounted on a glass slide beneath a long cover-slip supported by glass rods about 1 to 1.5 mm. in diameter. The slide is placed on the stage of a microscope in focus under a magnification of about 40 diameters and a drop of the solution to be tested is blown into the sperm suspension with a capillary pipette attached to a flexible rubber tube. The agglutination reaction begins within less than a second if the solution be of considerable agglutinating power, or within 2 or 3 seconds if the solution is diluted nearly to the unit reaction. In the latter case it lasts only 4 to 6 seconds. The masses formed are relatively small in the latter case, and larger and denser proportionately with the higher concentrations.

The reaction is accompanied by a strong stimulation of the spermatozoa, and the reversal of the reaction by slackening of movement, which is more or less proportional to the strength of the solution, so that after agglutination with a strong solution the spermatozoa are practically paralyzed for a time. For this reason the agglutination masses formed by a strong solution do not break up readily and it is difficult to fix the exact cessation of the reaction; but with concentrations up to about 20 units the beginning and end of the reaction are both clearly marked. For a more detailed account of the reaction with higher concentrations, see Study V (Lillie '13, p. 550).

As an important detail of technique, I would say that for the determination of the more delicate reactions it is desirable to have the sperm suspensions free from blood corpuscles or other foreign particles. I therefore proceed as follows: the sea-urchin is dried slightly with a towel and is opened by a circular cut on the oral side of the equator. If it be a ripe male the thick sperm will begin to exude from the genital pores. The animal is then

laid aboral side down in a Syracuse watch crystal and the sperm allowed to collect in the crystal. In this way, as much as 2 cc. of thick perfectly clean sperm may be obtained from a large ripe male. If a few drops of sea-water have dripped off with the sperm it may be removed with a pipette, but if the sperm is abundant it is so dense that the fluid lies on it without intermingling. Such a mass of sperm will keep perfectly fresh for several hours, and suspensions may be made from it as needed for the tests. But the suspension used in testing must be freshly prepared if delicate reactions are desired.

The fertilizin is actively secreted by the eggs in sea-water as noted beyond; the concentration in the fluid of a given suspension of eggs in sea-water is a function of the quantity of eggs in the suspension. It will be convenient, therefore, in referring to any given agglutination solution, to have a method of expressing the relative quantity of eggs in proportion to the sea-water. The most convenient way of expressing this relation is to express the bulk of eggs as a percentage of the entire suspension. Thus 1 cc. of eggs added to 9 cc. of sea-water will be denominated a 10 per cent egg suspension, etc. This form of expression was not adopted until after the experiments were done; the figures are, therefore, not always even numbers, and are not intended in any case to be absolutely exact, but this is a matter of little importance as the density of a given egg suspension is only one factor in the concentration of the fertilizin in it; the condition of the eggs, whether entirely ripe or immature, fresh or stale, with or without jelly, is more important.

2. THE QUANTITY OF FERTILIZIN PRODUCED BY OVA OF ARBACIA

a. By a single washing

July 6, 1913: For this experiment I used the eggs of a single female shed in a watch crystal in the same manner as described above for the collection of clean sperm. The eggs were thus free from blood or fragments of the ovaries, and contained only a trace of the sea-water. Graded quantities of eggs were put in a series of six test-tubes containing 5 cc. of sea-water each. The eggs were placed in the tubes at 9:45 A.M.; each tube was turned upside down six times and the eggs then allowed to

settle; there was no shaking or rough handling of the eggs, so that they were absolutely uninjured. At 10:20 A.M., after the eggs had settled to the bottoms of the tubes, samples of the sea-water in the tubes 1 to 5 were removed and tested by a series of dilutions; at 11:45 A.M. a sample of 6 was taken and tested. The concentration of the sperm agglutinating substance in each is shown in table 1.

TABLE 1

TUBE	EGG SUSPENSIONS PER CENT	AGGLUTINATING STRENGTH OF SUPERNATANT SEA-WATER	TESTS	
			Dilutions	Reactions
1	0.5	50	1/50	4–5 seconds
2	1.0	100	1/100	4–5 seconds
3	2.0	100+	1/100	10 seconds
4	4.0	400	1/400	6 seconds
5	8.0	800	1/800	6 seconds
6	16.6	1600	1/1600	7–8 seconds

It is interesting to note that the quantity of fertilizin produced is so nearly proportional to the quantity of eggs. (No. 3 would probably have tested to 1/200 with perfectly fresh sperm.) We might expect in general that the quantity of fertilizin produced would continue to be proportional to the quantity of eggs. This however, may not be the case, as there are indications that in the presence of a certain concentration in the sea-water more is not secreted by the eggs.

It would appear that after the eggs had settled in the tubes into a dense layer on the bottom they ceased to produce more agglutinating substance, because the concentration in tube 6 in which the sea-water had stood over the eggs much longer than in tubes 1 to 5 was proportional to the quantity of eggs and not to the time.

Higher concentrations of the fertilizin were often obtained: August 9: A 25 per cent egg suspension stood for 12 minutes. The supernatant fluid then gave the following sperm-agglutination tests: diluted to 1/6400, 7-second reaction; 1/3200, 15-second reaction. August 18: egg suspension (density not given) was shaken. After settling of the eggs the supernatant fluid

gave the following sperm-agglutination tests: diluted to 1/12800, 5-second reaction; 1/6400, 10 to 12-second reaction.

Enough has been said to indicate the large amounts of this substance produced by the eggs in sea-water. No attempt has been made to produce the highest attainable concentration. The standardization of solutions for experiments was a daily procedure and in the experiments themselves hundreds of tests were made.

Hitherto, I think, the unfertilized egg has been generally regarded as a very inert cell, almost in a state of suspended animation. But it is necessary to modify such a point of view very greatly. The egg of Arbacia, at least, is a very actively secreting cell. Although the method which I have used enables us to calculate only the relative concentration of the agglutinating substance produced, yet the quantity must be very considerable when we consider that in tube 1, of the preceding experiment the egg suspension was only 0.5 per cent and that the supernatant fluid was charged with 50 units of the agglutinating substance in 35 minutes.

b. Comparison of ripe and unripe eggs

The tests already given are from fresh, ripe eggs. Ovaries containing a high proportion of ovocytes produce less fertilizin proportionately; but such ovaries could be obtained only at the beginning of the season so that only a few tests were made. On June 16 I compared equal amounts of ripe ovaries with practically all eggs mature, and unripe ovaries with only 50 per cent of the eggs mature. One part finely cut up ovary was added in each case to 3 parts sea-water. The first tested to 1/612, the second to 1/64. In a second determination the same day, in which *a* had only 5 per cent unripe eggs (ovocytes with germinal vesicle) and *b* had 50 per cent, one part ovary to 2 parts sea-water in each case, the tests were *a* 1/1000, *b* 1/600. In the latter case the strength of the solutions was about proportional to the percentage of mature eggs. In the former case the unripe ovary did not give as large a production of the fertilizin as would be expected

from the relative proportions of ripe eggs. Presumably, the ovarian tissue was relatively more bulky in this case.

The ovocytes are surrounded with the same layer of jelly in Arbacia as the mature eggs; hence we must conclude that the jelly is not the source of the agglutinating substance to whatever extent it may become charged with it after maturation. An important point is involved in this conclusion discussed farther on.

I think we are justified in concluding that ovocytes do not secrete the agglutinating substance. The conditions in Nereis fortify this conclusion, inasmuch as the ovocytes with intact germinal vesicle do not secrete the fertilizin, but as soon as insemination take place it is poured out abundantly and the germinal vesicle breaks down.

In this connection it is important to note that in some animals ovocytes with intact germinal vesicle do not respond to the spermatozoon, even if cut in two so that the protoplasm is laid bare. Wilson ('03) has shown this for the nemertean, Cerebratulus, and Delage ('01) for the eggs of Strongylocentrotus and Asterias. As I show farther on that the fertilizin is necessary for fertilization, we may find in these facts evidence that the fertilizin is not only not secreted but is not even preformed in ovocytes with intact germinal vesicle, at any rate not in the forms mentioned. Its absence would furnish sufficient explanation of the failure of such egg-fragments to fertilize, and the consequent impossibility of producing merogony until the germinal vesicle breaks down.

Delage ('01) also shows that, as soon as the germinal vesicle begins to fade, in Asterias, and the nuclear sap diffuses into the cytoplasm, the egg becomes fertilizable and merogony can be induced. According to my interpretation the formation of fertilizin must begin at this time. At any rate the non-fertilizable condition of the cytoplasm before the germinal vesicle begins to fade is associated with the absence of fertilizin.

It is not necessary to assume that the fertilizin is contained in the nuclear sap; on the contrary, the long-continued production of this substance, as described in the next section, runs counter to this idea. It is more probable that it exists in a state comparable to zymogen ready to be converted into active form, and

that the escape of nuclear sap furnishes the necessary conditions. In some eggs, therefore, we might anticipate that sufficient substance might exude from the germinal vesicle without rupture to bring about formation of active fertilizin. Theoretically this should occur in such eggs as those of Nereis in which fertilization occurs before rupture of the germinal vesicle.

c. Fertilizin produced in a series of washings; rôle of the jelly

The production of this substance by the egg, in sea-water must be regarded as an active process of secretion, and, as will be seen immediately, it goes on continuously, in the case of Arbacia, as long as the eggs remain alive.

The last fact was ascertained in several attempts to remove all the fertilizin from the eggs by repeated washings. In these experiments it was also ascertained that while the jelly layer surrounding the eggs is saturated with fertilizin, eggs deprived of jelly still continue to produce it in considerable quantities. The method of making such tests is to suspend a measured amount of eggs in a given amount of sea-water and allow them to settle; as much of the supernatant fluid is then poured off as is possible without losing any eggs and kept for testing, and an equivalent amount of fresh sea-water replaced; the eggs are mixed up thoroughly, and allowed to settle, and the process is continued. In one series running three days in which the quantity of eggs was originally 2 cc. and the total volume of sea-water and eggs in the tube 10 cc., 6 to 8 cc. being poured off at each settling, thirty-four changes were made, and the agglutinating strength of the supernatant fluid diminished from 100 at first to 20 at the end. The details are given in table 2: Under the heading 'washings' the denominator gives the quantity of eggs and sea-water left after pouring off the supernatant fluid, and the numerator the total quantity after addition of the fresh sea-water.

This long series is amazing in its revelation of the extraordinary energy of the eggs in producing the fertilizin. In this case the eggs were allowed to retain their jelly, which, however, slowly dissolved until it was nearly all gone in washing 24.

TABLE 2

WASHINGS	TESTS	BULK OF EGGS	TIME OF CHANGE
1913			
August 14			
1 10/3.0 cc.	1/100—10–12 sec.	2.0 cc.	11:01 A.M.
2 10/2.5 cc.	1/100— 8 sec.		
3 10/2.5 cc.	1/50 —10–11 sec.	1.15 cc.	11:12 A.M.
4 10/2.0 cc.	1/50 — 10 sec.	1.1 cc.	11:22 A.M.
5 10/2.0 cc.	1/100— 6 sec.		11:32 A.M.
6 10/2.0 cc.	1/50 — 9 sec.	1.2 cc.	1:55 P.M.
7 10/1.7 cc.	1/100— 12 sec.	0.9 cc.	2:20 P.M.
8 10/1.7 cc.	1/100— 7 sec.		2:35 P.M.
9 10/1.7 cc.	1/100— 7 sec.		2:45 P.M.
10 10/1.7 cc.	1/100—11–13 sec.	0.8 cc.	2:53 P.M.
11 10/2.0 cc.	1/100— 11 sec.	0.8 cc.	3:03 P.M.
12 10/1.7 cc.	1/100— 8 sec.	0.8 cc.	3:13 P.M.
13 10/1.5 cc.[1]	1/100— 12 sec.		3:23 P.M.
14 Fluid not changed[2]	1/100— 11 sec.		4:12 P.M.
August 15			
14a 10/2.8 cc.	1/50 slow atypical reaction		8:18 A.M.
15 10/1.9 cc.	1/100— 12 sec.	0.7 cc.	9:20 A.M.
16 10/2.0 cc.	1/100— 13 sec.	0.7 cc.	9:55 A.M.
17 10/2.0 cc.	1/100— 7 sec.	0.65 cc.	10:22 A.M.
18 10/2.0 cc.	1/25 — 7 sec.		10:40 A.M.
	(Note sudden fall 17–18)		
19 10/2.0 cc.	1/50 — 5 sec.		11:04 A.M.
20 10/2.0 cc.	1/25 — 5 sec.	0.6 cc.	11:34 A.M.
21 10/2.0 cc.	1/20 — 9 sec.	0.5 cc.	2:15 P.M.
22 10/4.0 cc.	1/20 — 7 sec.		2:45 P.M.
23 10/ .0 cc.	1/20 — 7 sec.	0.55 cc.	3:45 P.M.
24 10/2.0 cc.	1/20 — 7 sec.		4:25 P.M.
No jelly left on the eggs, except a thin layer on a few			
August 16 Continued the above series to 33 washings from 9:08 A.M. to 4:05 P.M.			
25			
26			
27	1/20 — 7 sec.		
28			
29			
30[3]			
31	1/20 — 5 sec.		
32	1/10 — 10 sec.		
33	1/20 — 18 sec.		

[1] The jelly is now dissolved until only a thin layer is left. Washings 3–14 were absolutely colorless; and the following ones remained so until 35.

[2] The eggs were precipitated by slight centrifuging in the first 13 washings, except 6; in the others they were allowed to settle. The gradual reduction in bulk of the eggs is due to loss of jelly, not to loss of eggs.

[3] A drop from No. 30 was fertilized with excess of sperm at 11:36 A.M.; 5 per cent only segmented, irregularly; no membranes formed.

TABLE 2—Continued

WASHINGS	TESTS	BULK OF EGGS	TIME OF CHANGE
1913			
August 17			
34	12/1.5 cc.	1/20 — 5 sec.	0.325 cc.	9:15 A.M.
35	On stirring up, the eggs disintegrate, and the fluid becomes highly colored; the agglutinating substance is *absolutely neutralized*. Test 1/1 negative, but the fluid is highly chemotactic and activating.			

In order to determine the rôle of the jelly in charging the sea-water with fertilizin, I killed some eggs by heating them for 5 minutes to 60° C. (August 15, 1913,) and submitted them to a series of 22 washings in 3 days, by which time the jelly was nearly all dissolved away: the agglutinating power decreased from 800 at first to 1 at the end. The agglutinating substance could be detected as long as any jelly remained.

On the other hand, eggs may be entirely deprived of jelly by shaking, and such eggs will continue to produce fertilizin as long as eggs with jelly, though in lesser quantities at first. This matter is so fundamental that I include another long table giving a comparison of eggs deprived of jelly by shaking and those with jelly (table 3).

August 21, 1913: In this experiment ripe eggs of Arbacia were washed once and divided in three equal lots, *A*, *B*, and *C*, in 10 cc. sea-water in graduated cylinders. They were allowed to settle, and at 9:20 A.M. the bulk of eggs was 1.8 cc. in *A*, 2 cc. in *B*, and 2 cc. in *C*. Thus there were about 10 per cent fewer eggs in *A* than in *B* and *C*. At 9:23 *A* was transferred to a test-tube and given 6 vigorous shakes to get rid of the jelly. A sample was then examined in India ink and it was found that 90 per cent of the eggs were entirely freed from the jelly; in the remainder it was usually reduced to a thin layer. *A* was then returned to its cylinder in 10 cc. sea-water and allowed to settle. As the eggs settled in *A* it could be seen that the fluid was only slightly colored; so that it was certain that not many eggs were injured in the shaking.[2] At 9:40 A.M. the bulk of eggs was as follows: *A*, 0.6 cc.; *B*, 1.6 cc.; *C*, 1.6 cc. As care was taken to avoid loss of eggs in *A* the

[2] It should be noted that in such an experiment, it is extremely important not to shake too much, for if the eggs themselves are injured so that the color escapes, a complicating factor comes in considered beyond (see section 6, p. 544).

decrease in bulk is due to loss of jelly, in *B* and *C* simply to further settling. *C* was kept for control. *A* and *B* were submitted to repeated washings as in table 3, p. 536.

This experiment positively decides the question as to production of fertilizin. It continues to be produced by the unfertilized eggs without jelly as long as they live. The jelly is, naturally, saturated with fertilizin, and it seems probable that it may act to prevent too rapid exhaustion of the secreting mechanism of the egg. It is of course possible that all that the egg gives off is preformed, but it is more probable that loss of this substance by the egg acts as stimulus to regeneration of more.

Apparently the eggs do not secrete the fertilizin in such a series of washings after they have settled in a mass at the bottom of the tube; for it will be noticed that the concentration of the substance in the successive washings is not a factor of the time intervals. On the contrary, a long time interval between washings is often associated with a temporary decrease in concentration, as may be noted, for instance, in the interval of 15 hours between 14 and 14a in table 2. In this case 14 and 14a are actually the same body of supernatant fluid and the concentration is much decreased in the second test 15 hours after the first, as though the eggs were gradually destroying the fertilizin. This subject is fully treated in section 6 (anti-fertilizin).

3. THE BINDING OF FERTILIZIN BY SPERM

In my previous paper (Lillie '13) I showed that the spermatozoa fix the agglutinating substance, and assumed as a working hypothesis that the fixation was due to chemical union. The fact on which the statement was based was the disappearance of the agglutinating substance from a sperm suspension when not present in excess. No attempt was made, however, to make quantitative determinations, so the matter was taken up again in 1913 in a more exact manner. The amount of the agglutinating substance actually fixed by the sperm is surprisingly small when the great avidity of the sperm for it is taken into account.

TABLE 3

WASHINGS	TESTS	BULK OF EGGS	TIME OF CHANGE
1913			
August 21			
1 A 10/2.0		0.6 cc.	9:42 A.M.
B 10/3.0		1.6 cc.	9:42 A.M.
2 A 10/2.0		0.6 cc.	10:18 A.M.
B 10/2.4		1.4 cc.	10:18 A.M.
3 A 10/2.6		0.55 cc.	10:40 A.M.
B 10/3.0		1.4 cc.	10:40 A.M.
4 A 10/2.4		0.55 cc.	11:04 A.M.
B 10/2.7		1.3 cc.	11:04 A.M.
5 A 10/3.0		0.55 cc.	11:27 A.M.
B 10/2.6		1.2 cc.	11:27 A.M.
6 A 10/2.0		0.55 cc.	11:47 A.M.
B 10/2.7		1.2 cc.	11:47 A.M.
7 A 10/2.0		0.5 cc. plus	2:03 P.M.
B 10/1.6		0.8 cc.	2:03 P.M.
8 A 10/2.5	1/10 neg.; 1/1—50 sec.		2:30 P.M.
B 10/2.5	1/20—13 sec.		
9 A 10/2.0			3:15 P.M.
B 10/2.0			3:15 P.M.
10 A 10/2.0			3:45 P.M.
B 10/2.3			3:45 P.M.
11 A 10/2.0			4:06 P.M.
B 10/2.4			4:06 P.M.
Fertilization 1			
12 A 10/2.0			4:40 P.M.
B 10/2.5			4:40 P.M.
13 A 10/1.5			7:32 P.M.
B 10/1.2			7:32 P.M.
August 22			
14 A 10.4/3.4		0.4 cc.	8:18 A.M.
B 10/2.8		0.6 cc.	8:18 A.M.
15 A 10/1.4			8:38 A.M.
B 10/2.0			8:38 A.M.
16 A 11/1.8	1/10 trace; 1/1—60 sec.		9:13 A.M.
B 11/2.2	1/10—20 sec.		9:13 A.M.
Fertilization 2			
17 A 10/1.7			9:40 A.M.
B 10/2.5			9:40 A.M.
18 A 10/1.0			10:05 A.M.
B 10.5/2.4			10:05 A.M.
19 A 10/1.0			10:23 A.M.
B 10/3.0			10:23 A.M.

TABLE 3—Continued

WASHINGS	TESTS	BULK OF EGGS	TIME OF CHANGE
1913.			
August 22			
20 A 10/1.0	1/10 trace; 1/1 trace and strong chemotaxis		10:48 A.M.
B 10/2.4	1/10—9 sec.		
Note: Some eggs going to pieces in A			
21 A 10/1.5			11:10 A.M.
B 10/3.0			
22 A 10/1.0			11:30 A.M.
B 10/2.2			
23 A 10/1.4			2:12 P.M.
B 10/2 0			
24 A 10/1.4	1/1 trace and strong chemotaxis		2:33 A.M.
B 10/1.8	1/1—25 sec., slight chemotaxis		
25 A 10/1.0			2:55 P.M.
B 10/1.2			
26 A 11/1.0			4:03 P.M.
B 11/2.0			
27 A 10/1.0			4:19 P.M.
B 10/2.0			
Fertilization 3			
28 A 10/2 0			4:36 P.M.
B 10/1.0			
29 A 10/1.0			7:45 P.M.
B 11/2.0			
August 23			
30 A 10/1.0	1/1—20 sec., chemotaxis[1]		8:37 A.M.
B 10/2.5	1/1—30 sec., no chemotaxis		
31 A 10/1.5	1/1—14 sec. strong chemotaxis		9:13 A.M.
B 10/2.0	1/1—20 sec. strong chemotaxis		
Fertilization 4			
32 A 10/1.0			10:10 A.M.
B 10/2.0			
33 A 10/1.0	1/1—11 sec. strong chemotaxis		11:30 A.M.
B 10/1.6	1/1—11 sec. strong chemotaxis		

[1] The chemotaxis noted in this series is due to injured eggs which secrete antifertilizin and conceal the fertilizin. See discussion.

TABLE 3—Continued

WASHINGS	TESTS	BULK OF EGGS	TIME OF CHANGE
1913			
August 23			
34 A 10/0.5			2:36 P.M.
B 10/1.0			
35 A; fluid not changed	1/1—11 sec.		
B fluid not changed	1/1—10 sec.		
August 24			
36 A; fluid not changed	1/1—8 second reaction		9:35 A.M.
B fluid not changed	1/1—7 second reaction		
37 Eggs shaken	and broken; fluid not	changed.	9:42 A.M.
A	1/1—negative		
B	1/1—4 sec.; 2d test n	egative	

Thus on August 13, I standardized an agglutination solution in sea-water to about 128 agglutinating power and placed 1 cc. in a tube (A). To this was added 1 cc. of a 3.3 per cent sperm suspension; 1 cc. of the agglutinated mixture *A* was then added to tube (B) containing 1 cc. of the same sperm suspension; 1 cc. of *B* was then added to 1 cc. of the same sperm suspension in tube (C). Thus the 128 power agglutinating solution was diluted with 3.3 per cent sperm twice in *A*, four times in *B*, and eight times in *C*.

(A) 1 cc. 128 agglutinating power solution plus 1 cc. 3.3 per cent sperm
(B) 1 cc. A plus 1 cc. 3.3 per cent sperm
(C) 1 cc. B plus 1 cc. 3.3 per cent sperm

The sperm was then centrifuged off in the three tubes and on test the supernatant fluid was negative in *C*, faintly positive in *B* and gave a 60 to 80-second reaction in *A*. On renewed test, *B* was found almost negative. The result is then that 1 cc. of a 128 power agglutinating solution may be fixed by 3 cc. of 3.3 per cent sperm.

Other tests of the binding power of sperm gave results of the same order of magnitude. Thus on August 9, I used a 64 power agglutinating solution as follows:

(A) 1 cc. 64 agglutinating solution plus 1 cc. (approx.) 3 per cent sperm
(B) 1 cc. A plus 1 cc. 3 per cent sperm
(C) 1 cc. B plus 1 cc. 3 per cent sperm
(D) 1 cc. C plus 1 cc. 3 per cent sperm

In this case after centrifuging off the sperm, *B*; *C* and *D* were negative; *A* was faintly positive. That is, 1 cc. of approximately 3 per cent sperm fixed 1 cc. of a 64 power agglutinating solution. This shows a greater binding power than in the previous experiment, but easily within the limits of error arising from difficulty of standardizing the sperm suspension, owing to the unavoidable inclusion of some fluid in the sperm. Other tests gave comparable results. Reduced to common terms:

August 9: 1 cc. 3 per cent sperm fixes 1 cc. 64 power agglutinating solution.
August 13: 1 cc. 3.3 per cent sperm fixes 0.66 cc. 64 power agglutinating solution

The difference is considerable, but when one considers first the difficulty of obtaining equivalent sperm suspensions and second the 2 to 1 method of standardizing the agglutinating solutions, and third, possible variations in the delicacy of the sperm suspensions used as indicators, the results lie well within the limit of error and indicate that there is a definite quantitative relation involved.

The possibility remains that the disappearance of the agglutinating substance in sperm suspensions is a phenomenon of adsorption rather than of chemical union. Experiments planned for an adequate test of this possibility were crowded out by other problems.

If sperm suspensions are allowed to stand for some hours they reach a condition when they will agglutinate only with strong solutions. If the binding power is then tested it is found that they fix as much of the agglutinating substance as before. The fixing power of the sperm is thus entirely independent of its capacity for being agglutinated, which is of course to be expected. But it is important, nevertheless, to emphasize it; the capacity for being agglutinated depends upon the motility of the spermatozoa, the binding capacity depends merely upon the presence of

chemical side-chains (receptors) capable of uniting with the agglutinating substance. When such a union takes place the heads of the spermatozoa must be supposed to become adhesive; if they move about rapidly, there are more collisions and hence more agglutination. With stale spermatozoa, either the movements are too slow to produce much agglutination, or the receptors are cast off and lie free in the medium, so that their binding does not affect the physical properties of the spermatozoa themselves. The latter possibility should be investigated, but was not this summer owing to the multitude of other problems.

A little light may be thrown on this problem by the results of June 26: In that case 4 drops of a strong, but not definitely standardized, solution of the agglutinating substance was added to 4 cc. of a 3 per cent sperm suspension 3 hours old. The tube was then centrifuged strongly and most of the spermatozoa precipitated in a mass at the bottom; however the supernatant fluid remained opalescent. Tested immediately with a fresh sperm suspension the fluid gave a 17 to 25-second agglutinating reaction. The tube was allowed to stand five minutes longer and was then tested again; the precipitated sperm remained in a solid mass, but the supernatant fluid had become negative; it contained no more free agglutinating substance. The quantity of sperm in suspension seemed much too small to account for the neutralization, which I am inclined to attribute to free receptors. But no really adequate test was made.

As to the interpretation to be given to the reversal of agglutination of the sperm it may be supposed either, (1) that the adhesive condition on which the agglutination depends exists only during an early union of the agglutinating substance with the sperm receptors and is lost as the union becomes firmer; or (2) that the bound receptors are cast off by the sperm. In view of the nature of the mechanism of the fertilization process, discussed beyond, I am inclined to lean to the second hypothesis.

4. SEPARATENESS OF HETERO-AGGLUTINATING SUBSTANCES IN EGG-SECRETIONS

I stated in my previous paper that in strong agglutinating solutions from Arbacia eggs and ovaries there is present also a substance that agglutinates the sperm of Nereis, and I argued that this was probably distinct from the iso-agglutinating substance because (1) it is contained in the blood of Arbacia which is normally entirely free from the iso-agglutinating substance, and (2) it may disappear from agglutinating solutions on standing while the iso-agglutinating substance remains apparently intact.

Some farther experiments made this year demonstrate the fact that the substance in agglutinating solutions from Arbacia eggs, that attacks Nereis sperm is a separate substance. We may note in the first place that the effect of this substance on the Nereis sperm is distinctly toxic. A drop containing it injected into a Nereis sperm suspension beneath a raised cover slip causes the formation of a permanent coagulated ring at the margin of the drop. In this respect the action differs from the iso-agglutination which is without toxic effects.

In the second place, the quantity present in strong iso-agglutinating solutions of Arbacia is extraordinarily variable; thus on June 18, 1913, an agglutinating solution was made by addition of 3 parts sea-water to 2 parts cut-up ovaries and eggs of Arbacia. The test on Nereis sperm showed: (1) undiluted, formation of a solid ring-coagulum; (2) diluted one-half with sea-water, solid ring-coagulum; (3) diluted to one-fourth with sea-water, slight permanent coagulum; (4) diluted to one-eighth, small coagulated masses; (5) diluted to one-sixteenth, nearly neutral; (6) diluted to one-twenty-fifth, absolutely neutral. Acting on Arbacia sperm the same solution gave a 5-second reaction at 1/200 dilution. Other samples equally strong in the iso-agglutinating substance were nearly neutral for Nereis sperm undiluted.

In the third place it is possible to precipitate out all of the Nereis-active substance with Nereis sperm and leave the full complement of iso-agglutinating substance. Thus on June 19 an agglutinating solution was made by addition of 3 parts sea-

water to 2 parts ovaries and eggs of Arbacia; the fluid was filtered off. Undiluted it had a powerful effect on Nereis sperm but was negative at 1/16 dilution; 1 cc. was then placed in each of three tubes *A*, *B*, *C*. To *A* was added 2 drops of an aggregative Nereis sperm-suspension, to *B* 4 drops of the same, to *C* 8 drops of the same. Precipitates formed in all. On testing the supernatant fluids all were found to be negative to Nereis sperm. The hetero-agglutinating substance was completely fixed even in *A*. Tested for the iso-agglutinating substance, *A* was shown to be equal in power to the control. The tests gave:

(A) 1/800—faintest reaction
Control 1/800—faintest reaction
(A) 1/400—12 second reaction
Control—1/400—8 second reaction

Thus while all the Nereis-active substance was destroyed the iso-active substance remained in full amount.

This is of course conclusive; but I was also able to show that the sperm of Ctenolabrus (teleost) may precipitate the hetero-active substance leaving the iso-agglutinating substance intact. The hetero-active substance would therefore appear to be rather generally toxic to foreign sperm. It is present in greater quantity in the blood than in egg solutions as shown by definite experiments which need not be quoted in detail. It is rather probable, indeed, that its presence in the egg solutions is due to contamination by the blood. But I did not test this out fully, though it could readily be done by successive washings of the eggs.

A rather embarrassing fact that appeared in the course of the above tests is that Nereis sperm may destroy considerable quantities of the iso-agglutinating substance if present in excess of the amount required to fix the hetero-active substance. This came out first in the test of *C* (under June 19 above), which contained at least 4 times the amount of Nereis sperm needed to fix the hetero-active substance, for the supernatant fluid in this tube turned out to be several times less active in its iso-agglutinating properties than the untreated control. It looked as though the Nereis sperm would combine more actively with the iso-agglutinating substance than the Arbacia sperm itself.

There are, however, strong reasons against accepting this conclusion: (1) there is no visible effect of the iso-agglutinating substance on the Nereis sperm as should be the case if there were actual combination. (2) The action of the Nereis sperm is ferment-like, inasmuch as the iso-agglutinating substance continues to disappear progressively for a long period of time in the presence of excess of Nereis sperm, whereas this is not the case in the combination with Arbacia sperm. I shall therefore omit the details of the experiments under this head, being convinced that we are dealing here with a case of actual destruction of the iso-agglutinating substance and not with chemical combination.

5. SOME SPECIAL PROPERTIES OF THE FERTILIZIN

It would be interesting to know something concerning the chemical nature of this substance which is secreted by the eggs in such considerable quantities. This subject was, however, left for another occasion, as the investigation of its biological properties offered more problems than could be investigated at the time. Apart from these biological properties, all that I am now able to say about it is of a negative nature.

(a) It is colorless: Thus in table 2 all the washings after the first two were colorless though the concentration of the substance was considerable; similarly in table 3. (b) It must possess very considerable molecular size for it can be completely removed from any solution by filtering through a Berkefeld filter. Whether the solution be fresh or not, or of whatever concentration makes no difference; the filtrate obtained through a Berkefeld filter was invariably devoid of sperm agglutinating properties. On the other hand, it readily passes specially hardened filter paper. (c) Correspondingly it is non-dialyzable, not passing through the walls of celloidin tubes even in 24 hours. (d) It is extremely heat resistant being destroyed only slowly at the boiling point (see Study V, 1913). (e) As stated in my preliminary paper, I am indebted to Dr. Otto Glaser for the determination that it does not give the usual protein tests, even in the most concentrated solutions obtainable (see Glaser, 1914a) It is

thus colorless, non-filterable, non-dialyzable, very thermo-stable and apparently non-protein in its character.

The stability of the substance is also evidenced in its lasting qualities. Filtrates from egg-suspensions were kept and tested after various intervals of time. Thus the filtrate from a 33 per cent egg-suspension of June 21, was of 1600 agglutinating power; on August 26 it was of 100 agglutinating power. Similarly, the filtrate from a 33 per cent egg-suspension of June 20 was of 1600 agglutinating power; on August 26 it was of 100 agglutinating power. On the other hand, an agglutinating solution of June 30, of 800 agglutinating power was practically negative on August 26. In this tube there was more evidence of bacterial action than in the other two. Again, a distilled water extract kept from September, 1912, was of 16 agglutinating power on July 7, 1913.

In Study V, I also noted that its production is confined entirely to the eggs. It is not contained in the blood, and could not be extracted from other tissues.

6. ANTI-FERTILIZIN

We have noted the extreme avidity of the spermatozoa for the fertilizin produced by the eggs. Neither the blood nor tissues appears to contain any substance capable of uniting with the spermophile side-chain which causes agglutination of the spermatozoa, or of neutralizing its action. However, such a substance is contained within the egg itself, and its discovery is one of the most interesting points in the mechanism of fertilization, because it would appear from the facts considered in section 7 that it is the occupancy of the spermophile side-chain of the fertilizin by this substance, which I propose to call anti-fertilizin, that prevents polyspermy, or refertilization of eggs.

Its presence can be demonstrated in the test-tube by methods which extract the internal substance of the eggs. But as the jelly surrounding the eggs contains an immense excess of fertilizin, complete neutralization of sperm agglutinating power can be obtained only after the jelly is removed, and still better if the eggs have been repeatedly washed until their fertilizin production is greatly reduced.

To consider the latter method first: The matter was first brought clearly to my attention in the experiment listed in table 2. In the 34th washing the supernatant fluid tested to 1/20 dilution; it was poured off, leaving 1.5 cc. in the tube, and 10.5 cc. of sea-water was added. When the eggs were stirred up they at once disintegrated, liberating the red pigment so that the fluid which in previous washings had been colorless became bright red. When this fluid was tested with a fresh sperm suspension it was absolutely without agglutinating effect. The dilution of the 20 power fluid of the 34th washing was 8 times, so that even if the eggs had suddenly ceased the production of the agglutinating substance the fluid would have contained 2.5 agglutinating units and should have given at least a 10-second agglutination reaction; but it was absolutely negative. The agglutinating substance had therefore been neutralized by some substance escaping from the disintegrating eggs.

In the experiment listed in table 3, the repetition of this result was of course looked for. On the morning of August 24, after 35 washings on previous days, I noticed that pigment was beginning to diffuse from the eggs in tubes *A* and *B*, but it had not extended above the 1.5 cc. mark. The eggs were beginning to break down. Without disturbing them, I tested the colorless supernatant fluid and got undiluted an 8-second reaction in *A* and a 7-second reaction in *B*. Both tubes were then vigorously shaken until the eggs were disintegrated and the fluid colored with the escaping pigment. Tested in 4 minutes *A* was negative for agglutination, *B* gave a 4-second reaction. Tube *B* was again shaken and in 11 minutes more its agglutinating action had also disappeared.

There can, therefore, be no doubt that the egg contains in its interior a substance that unites with the spermophile group of the fertilizin.

In these experiments it was interesting to note that after the agglutinating action had disappeared, the solution was very strongly chemotactic, so that the drop injected into the sperm suspensions formed a ring of active, entirely unagglutinated spermatozoa. The chemotactic substance is certainly distinct

from the fertilizin. The same observation was made also on other occasions.

The presence of the anti-fertilizin in the eggs could also be demonstrated in extracts of fresh eggs. These were made (1) by extracting in distilled water, (2) by drying eggs and grinding them in sea-water, (3) by grinding fresh eggs with sand in a mortar. I had previously often noticed that such extracts gave decidedly atypical agglutination of the spermatozoa, as compared with the action of the secretion of the eggs into sea-water. Distilled water extracts were then examined to find the cause for such atypical behavior. After it became clear from the experiments quoted above that the eggs contained the anti-fertilizin, the method of making the distilled water extracts was modified by first shaking the eggs as free from jelly as possible, so as to avoid the great excess of fertilizin contained in the jelly. These experiments yielded the following results:

August 22, 1913: 10:35 A.M. washed eggs were shaken six times in a test tube to get rid of the jelly. The eggs were washed twice again and shaken six times more; 60 per cent lost jelly; let stand. 2:15 P.M. Poured off sea-water to 2 cc. and added fresh sea-water to 8 cc. and divided equally in two test tubes, *A* and *B*; allowed to settle. 2:27 P.M. Poured off sea-water from settled eggs to 1 cc. in both *A* and *B*. To *A* added 7 cc. sea-water. To *B* added 7 cc. distilled water. The eggs were allowed to stand until they were completely plasmolyzed in *B* and the supernatant fluid was bright red. Tests were then made within an hour, or less, of the supernatant fluid in *A* and *B*, for the fertilizin, with somewhat surprising results:

(*A*) *sea-water secretions* *Dilution tests*	(*B*) *distilled water extract* *Dilution tests*
	1/1280—faint trace
	1/640 —7 sec. reaction
	1/320 —12 sec. reaction
1/160—negative	1/160 —12 sec. reaction
1/80 —negative	1/80 —12 sec. reaction
1/40 —negative	1/40 —10 sec. reaction
1/20 —6 sec. reaction	1/20 —14 sec. reaction (strong chemotaxis)
1/1 —90 sec. reaction	1/1 —12 sec. reaction (strong chemotaxis)

Thus if only extreme dilutions were compared the conclusion would be that *B* is 32 times stronger than *A*, whereas comparison of the undi-

luted fluid would lead to the conclusion that *A* was 7½ times stronger than *B*. The fact that the time of the reaction of the *B* extract is about the same at all concentrations is in most pronounced contrast to the sea-water secretions in a very large number of tests, which had shown that the duration of the reaction is about proportional to the concentration of the agglutinating substance. The inference from the fact would be that the extract (*B*) contained an inhibitor for the agglutinating reaction which is diluted equally with the agglutinating substance. This might be a substance entering into combination with the agglutinating substance, but slowly and loosely at first.

4:07 P.M. Tube *B* was shaken vigorously to complete the plasmolysis. Tube *A* was stirred up. Both were then left until the next morning.

August 23, 1913: Tests of agglutinating power on fresh sperm:

Tube A	*Tube B*
Dilution tests	*Dilution tests*
1/20— 6 sec.	Negative at all dilutions 1/1280, 1/640, 1/320, 1/160, 1/80, 1/40, 1/20, 1/10, 1/1, but showing decided chemotaxis.
1/10—12 sec.	
1/1 —190 sec.	

Thus the neutralization of the fertilizin had become complete in the intervening time.

The disappearance of the agglutinating substance is not due to any destructive effect of distilled water on it, for control experiments showed that this was not the case.

A similar experiment on August 20 gave even more clear cut results. In this case (1) the eggs were deprived of jelly by 6 shakes, and examination of the eggs in ink showed that jelly was almost completely removed. (2) The eggs were allowed to settle and the supernatant fluid poured off. (3) The eggs were then divided into two equal parts in test-tubes *A* and *B*. (4) To *A* added 9 cc. fresh sea-water, to *B* added 9 cc. distilled water. (5) Each was stirred up twice and stood 30 minutes.

Tested with fresh 1 per cent sperm suspension, *A* gave a 10 second reaction at 1/40 dilution. *B* was negative at 1/2 and gave a faint reaction only, undiluted. Thus as *B* must have contained as much fertilizin as *A*, its disappearance must have been due to escape of antifertilizin by plasmolysis, inasmuch as we know that distilled water does not destroy it.

It would follow from this experiment in which the jelly was practically entirely removed, that the eggs contained an amount of antifertilizin sufficient to neutralize all the fertilizin present in them.

A third experiment yielded results similar to the first:

August 23, 1913: Fine lot of fresh eggs; shaken 6 times, 2:15 P.M. in test tube. Examination in ink showed about half have entirely lost

jelly, and that it is much reduced in nearly all. About 2 per cent of the eggs broken.

2:33 P.M. washed: Divided in two equal lots in test-tubes *A* and *B*. 3:08 P.M.: After settling of eggs poured off sea-water down to 2 cc. in each tube. To *A* added 6 cc. sea-water. To *B* added 6 cc. distilled water. Stirred several times until *B* was thoroughly laked.

Tests with fresh 1 per cent sperm

A. 3.40 P.M.	B. 4.05 TO 4.15 P.M.	B. 4.35 P.M.
	1/640— 6 sec. reaction	1/640— 6 sec. reaction
	1/320— 7 sec. reaction	1/320— 8 sec. reaction
	1/160— 10 sec. reaction	1/160—12 sec. reaction
1/80— 4 sec. reaction	1/80 — 36 sec. reaction	1/80 —25 sec. reaction (chemotaxis)
1/40— 8 sec. reaction	1/40 — 60 sec. reaction	1/40 —22 sec. reaction
1/20— 13 sec. reaction	1/20 —110 sec. reaction	1/20 —25 sec. reaction
1/10— 17 sec. reaction (1)	1/10 — 22 sec. reaction	1/10 —50 sec. reaction
1/1 —130 sec. reaction (1)	1/1 — 75 sec. reaction	1/1 —90 (?) sec. reaction

The second test of *B* indicated some progress in the neutralization of the fertilizin (cf. 1/20, 1/40, 1/80). The *B* dilution series is extremely irregular. Reading back from 1/1 (first test) the big jump from 1/10 to 1/20 indicates a breaking of the fertilizin X anti-fertilizin combination at this dilution.

August 24: Test showed some fertilizin present in *B*. Having poor sperm for indicator, left until next day. Shook up *B* vigorously; stirred *A*.

August 25: *B* now negative; *A* strongly agglutinative.

The distilled water extracts thus show the presence of a substance which combines quickly with the spermophile group of the fertilizin. In the experiment where the jelly was all removed by the preliminary shaking, there was practically immediate neutralization of the fertilizin. In the first and third, where the fertilizin was present in excess owing to the presence of some jelly, the excess was only slowly combined. Experiments with extracts of dried eggs (August 26) and with destruction of eggs by shaking or grinding also showed the presence of anti-fertilizin.

7. THE NECESSITY OF FERTILIZIN FOR FERTILIZATION

It will be apparent from the preceding facts and considerations that the fertilizin answers some of the requirements of a binding link between ovum and spermatozoon. When we consider the extraordinary activity of the unfertilized egg in its secretion, and the equally extraordinary avidity of the spermatozoa for it, one cannot escape the conviction that it must be a link in the normal fertilization process. When, moreover, one finds that the egg contains a more centrally located substance that can occupy the same combining group as the sperm, one seems to have before one, a view of a mechanism for preventing polyspermy.

I adopted, then, the working hypothesis that this substance is necessary for fertilization and there followed immediately three corollaries, viz: (1) If it were possible to extract this substance from eggs, they should no longer be capable of fertilization; (2) fertilized eggs are incapable of uniting again with spermatozoa, hence, if the hypothesis is correct, they could no longer contain free fertilizin; (3) eggs in which membranes have been formed by methods of artificial parthenogenesis become incapable of fertilization; such eggs must also, therefore, be devoid of free fertilizin after they have reached the non-fertilizable condition if the hypothesis is correct. These consequences of the theory were actually found to be true.

a. Fertilization of washed eggs

The only way of which I could think of extracting the fertilizin from the eggs without injury, is the method of repeated washings. But we have seen (tables 2 and 3) that it is actually impossible to remove all the fertilizin from a mass of eggs by any number of washings, for the eggs continue to produce it until they go to pieces However, the quantity produced in successive washings slowly diminishes. Therefore, we might expect on the basis of the theory, that there would be a gradual reduction of the percentages of eggs fertilized after many repeated washings. This actually occurs, as table 4 shows. It might also be anticipated that as

the quantity of fertilizin decreases, the developmental energy of the fertilized eggs might decrease, owing to relative incompleteness of fertilization. This also occurs. In table 3 (p. 536) it will be seen that fertilizations were made at the times of the 11th, 16th, 27th and 31st washings. In each case about three drops of both egg-suspensions *A* and *B* were taken, including several hundred eggs and fertilized with considerable excess of fresh sperm. It will be recalled that the eggs of lot *A* had been deprived of their jelly by shaking, whereas those of lot *B* retained the jelly. *A* lost its fertilizin relatively rapidly therefore, at first, but later the repeated washings caused removal of all the jelly in *B* also.

Table 4 shows the fertilization results, as regards the percentage of eggs segmenting (200 eggs were counted in each case). There is thus the anticipated decrease in percentage of fertilizations. Moreover, the time between insemination and the appearance of cleavage increases very much, and the eggs cease after a while to produce fertilization membranes and the cells tend to fall apart.

TABLE 4

DATE		NUMBER OF WASHINGS	
August 21	4:06 P.M. Fert. 1	11	A 76.0% segmented
			B 97.5% segmented
August 22	9:13 A.M. Fert. 2	16	A 76.0% segmented
			B 92.0% segmented
August 22	4:19 P.M. Fert. 3	27	A 83.5% segmented
			B 97.5% segmented
August 23	9:13 A.M. Fert. 4	31	A 39.0% segmented
			B 30.0% segmented
August 23	11:30 A.M. Fert. 5	33	A 25.0% segmented
			B 24.0% segmented

Concomitantly, with these effects of the series of washings, the developmental energy becomes greatly reduced. This was very obvious from the second fertilization. On August 24 (48 hours after fertilization) a large quantity of living material was contained in the second *A* fertilization, but not one had even approximately pluteus structure. The most common form was a

stereoblastula. In the second *B* fertilization there were a few abnormal prismatic plutei, while the majority were gastrulae; some stereoblastulae. The third fertilization resulted in extremely abnormal ciliated types. The fourth and fifth did not proceed beyond abnormal cleavage stages.

The eggs have evidently lost something which affects their power of fertilization. Table 3 shows the measure of loss of the sperm-agglutinating substance, and justifies the general conclusion that this is a factor in the result. The loss of other substances may also combine in the decrease of fertilizing power, but of this we know nothing definite. As a matter of fact, fertilizing power is gradually lost with decrease of fertilizin content of the egg.

A considerable number of eggs go to pieces during the washings. This was especially noticeable in the *A* series of the above experiment. Thus after the 19th washing my notes record that eggs were stuck to the tube to the 3.5 cc. mark, whereas in *B* where the eggs were protected by jelly, only a very few stuck and these did not extend above the 1 cc. mark. Such eggs liberate antifertilizin, and if this is present in sufficient amount it may conceal the fertilizin production of the intact eggs. This phenomenon appeared a number of times in my earlier experiments when eggs were shaken too strongly in the effort to get rid of the jelly, resulting in injury to a large proportion of eggs, which soon broke down and neutralized the fertilizin produced by intact eggs.

One other experiment with apparently much more decisive results may be given (Aug. 18, 1913). In this experiment the shaking lasted 45 seconds and over 99 per cent of the eggs were stripped of jelly. The eggs were then precipitated by the centrifuge using just enough force to ensure complete precipitation, and the supernatant fluid poured off. It was very highly colored, showing that many eggs had been broken. It tested to a dilution of 1/12800, giving a 5 second reaction. The eggs were then repeatedly washed about 10/1.5 each time. The time of change and tests of supernatant fluids are shown in table 5, p. 552.

3:45 P.M. The fluid in 15 now gives a 6 second reaction, showing that some eggs are still producing fertilizin, but that it is rapidly neutralized.

After the 8th washing these eggs had extremely little capacity for fertilization, although only 24 hours old: Fertilization 1. 9:08 A.M. August 19: A few drops of stirred up egg suspension put in 9 cc. seawater and 3 drops 1 per cent sperm added. This is an enormous excess

TABLE 5

WASHING	TIME	TEST
2	2:48 P.M.	1/6400
3	3:15 P.M.	1/800
4	3:47 P.M.	1/100 (bulk of eggs 0.2 cc.)
5	4:05 P.M.	1/20
6	5:25 P.M.	1/10
7	Aug. 19: eggs had stood in tubes over night	
	8:37 A.M.	1/10 negative. 1/1—13 seconds
8	9:10 A.M.	1/10—11 seconds
9	9:42 A.M.	1/10—10 seconds
10	10:00 A.M.	1/10—(faint)
11	10:20 A.M.	1/1 —11 sec. (strong chemotaxis)
12	10:47 A.M.	1/1 — 9 sec. (strong chemotaxis)
13	11:08 A.M.	1/1 — 5 sec.
14	11:53 A.M.	1/1 — 4 sec.
15	2:00 A.M.	1/1 — (negative)

of sperm. The eggs were rapidly literally covered with a single layer of adherent spermatozoa, but in spite of this only about 1 per cent divided (out of 200 eggs counted 3 were divided irregularly at 11:15). Some fresh eggs fertilized for control of sperm at same time showed practically all eggs divided at this time. Fertilization 2. 10:02 A.M. Same as 1, with control: 2:03 P.M. About 1 per cent divided; irregular, delayed; 80 per cent of control. Fertilization 3. 11:02. Same as 1, with control: 2:15 P.M. About 2 per cent divided; control 99 per cent divided.

Thus these eggs had almost entirely lost the capacity for fertilization with their loss of fertilizin, although they were only 24 hours in the sea-water. However, I regard the result given in the first experiment under this head as more typical for mere loss of fertilizin. In the experiment just quoted, the eggs had been injured to such an extent that they were liberating anti-fertilizin also, and I believe that this combined with the fertilizin in the cortex of the egg so as to block fertilization in this way. The extraordinarily rapid decrease of agglutinating power of successive washings (1, 12,800; 2, 6400; 3, 800; 4, 100; 5, 20; 6, 10; 7, 2 (?); 8, 10; 9, 10; 10, 10; 11, 1; 12, 1; 13, 1; 14, 1; 15, 0) is such as occurs only with injured eggs.

Other experiments give similar results. In one of these, the fertilizing power was reduced to about 1/8 in the third washing

after violent shaking which removed the jelly and in which the agglutinating power had been reduced from 400 to 10. It would appear from this that it makes no difference how rapidly we dispose of the fertilizin; the eggs are no longer capable of fertilization when it is gone.

b. Cessation of fertilizin production after fertilization

In a previous paper (Lillie '13) I noted that eggs of Nereis cease to produce sperm-agglutinating substance soon after fertilization, but that apparently this was not the case in Arbacia. I, however, ventured the suggestion that the apparent continued production of the sperm-agglutinating substance in Arbacia might be due to the presence of unfertilized eggs, of which there was a considerable percentage in the cultures. This may have had something to do with the results there described, but the factor that leads to an apparent continuation of fertilizin production in fertilized eggs is its presence in high concentration in the jelly, a condition that I, by no means, realized at first. When this factor was eliminated it was easy to demonstrate that fertilized eggs no longer produce the substance. The crucial experiments were as follows:

Experiment 1: August 23, 1913: A large quantity of unusually fine eggs were washed three times and fertilized at 2:30 P.M. with considerable excess of sperm. At 2:40 P.M. microscopical observation showed that nearly all had beautifully formed fertilization membranes. About 10 per cent of the eggs were without jelly (observation in ink). These usually had as fine membranes as the others.

The eggs were divided in two equal quantities in test tubes *A* and *B*. 2:50 P.M.: *B* was shaken 6 times to get rid of the jelly. Examination in ink showed that the jelly was removed completely from practically all the eggs. *A* (with jelly left on eggs) and *B* were then submitted to a series of washings with tests for fertilizin as shown in table 6, p. 554.

Thus in five washings the *B* fertilized eggs without jelly no longer gave the fertilizin test. This number of washings would be only barely sufficient to remove the agglutinating substance present before fertilization and the loose jelly. Two successive negative tests were given, showing that the production of the agglutinating substance has ceased absolutely. The *A* eggs were

TABLE 6

3:10 P.M.	A 1	Washed 8/2.0	
	B 1	Washed 8/1.0	
3:25 P.M.	A 2	Washed 8/1.5	
	B 2	Washed 8/1.0	
3:43 P.M.	A 3	Washed 8/2.5	
	B 3	Washed 8/1.0	Test 1/1—22 seconds
4:08 P.M.	A 4	Washed 8/2.0	
	B 4	Washed 8/1.0	Test 1/1— 9 seconds
4:53 P.M.	A 5	Washed 8/1.5	Test 1/1 over 75 seconds
	B 5	Washed 8/1.0	Test 1/1—negative
4.58 P.M.	A 5		Second test 1/1—60 seconds
	B 5		Second test 1/1—negative

apparently still producing it vigorously, but that is only because they carried the fertilizin-saturated jelly with them. Ninety-eight per cent of the *B* eggs segmented perfectly.

The result is in accord with the assumption that all free fertilizin is fixed *at the moment of fertilization*, or membrane formation. But I know of no way of absolutely demonstrating that the fixation takes place thus rapidly. But if such fixation is the mechanism for prevention of polyspermy, as I believe, the fixation of all free fertilizin must take place in a few seconds at most.

A duplicate experiment was made on August 25. The methods of Experiment 1 were repeated exactly. The eggs were not, however, so good a lot and the membranes did not stand put so far from the eggs. The shaking of lot *B* removed the jelly from most, but not from all. In this experiment tube *B* came negative on the sixth and seventh washings; whereas tube *A* gave a 90-second reaction, undiluted, on the sixth washing. Over 98 per cent of the eggs segmented and the unsegmented eggs had spindles.

Both experiments were clear-cut; there can be no question that after fertilization the eggs cease to produce fertilizin. Two effects are involved; the disappearance of fertilizin, and failure to produce more. The first effect might be due to extrusion of all fertilizin from the eggs; but it seems improbable that this could take place with sufficient rapidity to prove effective as a mechanism for preventing polyspermy, and inasmuch as we know that the eggs contain anti-fertilizin capable of occupying the spermo-

phile group of the fertilizin, it is more reasonable to conclude that the disappearance of sperm-agglutinating power from the washings of fertilized eggs deprived of jelly is due to neutralization of the substance remaining in the egg. In this connection it is instructive to note that eggs commonly lose pigment on insemination; I always have found the anti-fertilizin accompanied by pigment; it is, therefore, natural to suppose that anti-fertilizin is carried to the surface of the egg on insemination, thus affording the opportunity for combination with the fertilizin and its neutralization.

The other possibility that the sperm combines with all the free fertilizin in the egg appears to me inadmissible, because (1) the surface of the egg is relatively so enormous, and (2) the amount of fertilizin which the sperm is capable of binding is very small relative to the amount produced by the eggs even at a single washing (cf. sec. 3, p. 538). The bulk of the sperm actually used for insemination is insignificant compared to the bulk of the eggs, and only a small proportion of the sperm used actually fertilizes so that the amount fixed by the sperm itself is excessively minute compared to the quantity contained in the egg.

We have seen (p. 529) that 1 cc. of eggs charges 5 cc. of seawater to 1600 units. We have also seen that such eggs contain or produce many times this quantity in successive washings; and that it takes 1 cc. of 3 per cent sperm to fix 1 cc. of 64 power agglutinating solution. Thus it would require 1 cc. $\times 1600/64 \times 5$ or 125 cc. of 3 per cent sperm to neutralize the agglutinating substance produced by one washing of 1 cc. of eggs in 5 cc. of seawater. But a single drop of 3 per cent sperm would be much more than sufficient to fertilize the same amount of eggs. The disproportion is even greater than indicated in this rough calculation, which is, however, sufficient to show the absurdity of explaining neutralization of the agglutinating substance of the fertilized eggs by the sperm used in fertilization.

Theories of prevention of polyspermy, or of the non-fertilizable character of eggs already fertilized, proceed along two lines: (1) that the membrane formed on fertilization is impermeable to spermatozoa, (2) that the protoplasm of the fertilized eggs has

undergone some 'physiological' change which prevents union with the sperm. The first theory is an old one based on conditions in the sea-urchins; it is inapplicable to the ova of most animals, which do not form special membranes after fertilization; whatever value the fertilization membrane may have in special cases as guarding against polyspermy, it has been shown for some cases at least that the real cause of failure of refertilization is more deeply seated. Thus Wilson ('03) shows for Cerebratulus that after the germinal vesicle has faded, cytoplasmic fragments devoid of nucleus may be fertilized and undergo typical merogonic development; but enucleated fragments of fertilized eggs are incapable of refertilization: "Even when such fragments are placed in water containing sperm immediately after the section before they have assumed a spherical form, they fail to fertilize, though spermatozoa may be observed adhering to their periphery." Wilson rightly attributes the sterility of such fragments to a physiological change of the cytoplasm.

The theory of a physiological change of the cytoplasm following fertilization has been generally accepted, but there has been no idea of the nature of the change involved. Wilson directs attention to the similar sterility of egg fragments prior to fading of the germinal vesicle and suggests that the cytoplasmic change following fertilization is physiologically the opposite of that following the fading of the germinal vesicle. Now, as I have shown the probability that the agglutinating substance is not present in an active form in Arbacia prior to fading of the germinal vesicle, and that it becomes neutralized by union with anti-fertilizin following fertilization, it is clear that this mechanism answers the requirements of the problem. The fundamental mechanism for the prevention of polyspermy is the neutralization of the fertilizin by the anti-fertilizin present in the egg; i.e., the occupancy of the spermophile side-chain of the fertilizin by the anti-fertilizin.[3]

[3] Note: Delage ('01) determined for Asterias glacialis that fragments of the ovocyte with intact germinal vesicle are sterile, so that merogony cannot be produced. As soon as the germinal vesicle fades fragments are fertilizable and merogonic development may follow. Wilson's observations are in accord with

The question may be raised why such neutralization of the fertilizin is delayed until the moment of fertilization? The answer to this difficulty is fairly clear. The fertilizin is located in the cortex of the egg, and the anti-fertilizin is more deeply situated; they therefore do not interact so long as the cell body as a whole is quiescent. But as soon as the cortical fertilizin becomes activated by union with the sperm it at once begins to attack certain substances in the egg, as demonstrated in the third part of this paper; this sets up diffusion evidenced by escape of pigment, and by cytoplasmic flowing, and the two substances are brought together and interact. While this explanation is partly hypothetical, the spatial separation of the fertilizin and anti-fertilizin and the quiescent character of the cell-body in the unfertilized egg are facts; so also are the movements of diffusion and the cytoplasmic currents set up on fertilization. However it is possible that some other factor is operative in the interaction of fertilizin and anti-fertilizin following fertilization, which has entirely escaped attention.

c. The cessation of fertilizin production after formation of membranes by butyric acid

Some methods of artificial parthenogenesis cause eggs to become non-fertilizable; others apparently do not. In the sea-urchins it would seem that those methods that cause membrane formation result in the non-fertilizable condition while those which do not, need not have such an effect. Thus the use of hypertonic sea-water causes a certain amount of parthenogenesis

this as noted. But Delage farther asserts that a second change occurs after the formation of the second polar globule so that non-nucleated fragments again become sterile, although entire eggs can still be fertilized. This result is unsupported so far as I know, and would be diffcult to explain on the basis of the mechanism described in this paper except on the assumption that the original contribution of nuclear sap necessary for the production of fertilizin has become exhausted, and that presence of the nucleus had become necessary for continued production of fertilizin. Delage shows that during the fertilizable period of the cytoplasm when merogony can be effected parthenogenetic agents are especially effective, a result that agrees particularly well with my point of view.

in sea-urchin eggs without membrane formation but the development tends to be quite abnormal. Loeb has observed that membrane formation may be induced in such eggs after they have reached even the 8 or 16-celled stage by addition of sperm; they are still fertilizable, so far at least as the cortical changes are concerned. It is fair to assume in such a case that the lack of membrane formation is evidence of persistence of free fertilizin in the cortex, for the only cases in which I found this substance absent from mature eggs are eggs with membranes formed. This case, therefore, need offer no difficulty for the contention that the fertilizin is necessary for fertilization.

On the other hand, Loeb's method of treatment with fatty acids leads to membrane formation and such eggs are non-fertilizable, as Loeb states and I have myself observed. Loeb, however, goes on to assert that if such eggs are shaken so as to destroy the membrane and if sperm is then added, a new membrane is formed and the typical development of sperm-fertilized eggs follows. This would seem at first sight to run counter to my theory unless it could be shown that membrane-formation by butyric acid differed from membrane formation by sperm in not causing cessation of fertilizin production. However, as I shall show immediately, the production of fertilizin does cease after the formation of good membranes by butyric acid.

It is necessary, therefore, to examine Loeb's statement rather carefully. It is as follows:

> Dr. Kupelwieser und ich stellten diesen Versuch an, indem wir die Membranbildung bei unbefruchteten Eiern mittels Buttersäure hervorriefen, diese Membran dann *unmittelbar nach Bildung derselben*, durch Schütteln der Eier zum Platzen brachten und *sofort* Samen zufügten. Die Eier bildeten eine neue *dem Zytoplasma dicht anliegende Membran* und furchten sich mit einer der Samenbefruchtung entsprechenden Geschwindigkeit, und Zwar zunächst ausnahmslos in zwei Zellen. (Loeb '09, p. 210; italics mine).

It is implied that such superposition of fertilization on parthenogenesis would not take place unless the experiment was performed immediately after membrane formation. Presumably some fertilizin remained unbound for a short time and on account

of its relatively small amount the new membrane was formed close to the egg. The implied fact that the experiment failed after a short time supports my theory of the function of fertilizin, and the stated result is not at all inconsistent with my views. According to my point of view the possibility of superimposing fertilization on parthenogenesis would persist so long as a sufficient quantity of fertilizin remained unbound, i.e., so long as the reaction was incomplete.[4]

The non-fertilizable character of such eggs implies on my theory an absence of free fertilizin. This condition obtains, as the following experiments show:

Experiment August 26, 1913: A quantity of fresh Arbacia eggs were taken and some were set aside in a test tube as control (0.14 cc. when settled). The balance were concentrated in 3.4 cc. sea-water.

3:47 P.M. The latter were then added to 25 cc. butyric acid in sea-water made by adding 2.8 cc. $\frac{N}{10}$ butyric acid to 50 cc. sea-water.

3:47 1/2 P.M. Half of the eggs with acid were poured into 1500 cc. sea-water in crystallization dish *A* and stirred up to stop the action of the acid.

3:48 P.M. The remainder were poured into 1500 cc. sea-water in crystallization dish *B* and stirred up. *B* was over-exposed and the eggs agglutinated heavily. In *A* there was no agglutination and nearly all of the eggs formed fine membranes. The jelly was absolutely all gone owing to the action of the butyric acid, so there was no need to shake them.

The eggs in *A* and *B* were then concentrated, and placed in graduated tubes similar to the control. When settled there was 0.3 cc. eggs in each, estimated to be at least 4 times as many as in the control tube when allowance is made for the bulk of the jelly in the control. All were then submitted to successive washings with tests as shown in table 7, p. 560.

Thus the *A* eggs, with membranes formed, came negative in four washings, at least as rapidly as fertilized eggs, whereas the unfertilized control with fewer eggs was still showing an agglutination strength of about 10 (control 4, 1/1—50 seconds). There

[4] The essential problem is whether fertilization is of the nature of an irreversible chemical reaction, as I claim, or is merely a physical surface effect. Although this question has been investigated a good deal, it is still far from being definitively settled. The students of artificial parthenogenesis take the latter point of view generally.

TABLE 7

	WASHINGS	TESTS FOR FERTILIZIN
4:13 P.M.		
Control 1	10/1.2	
4:30 P.M.	A 1 12/2.4	Test 1/10—11 seconds
	B 1 12/1:5	
Control 2	11/2.0	Test 1/1 —2 plus min.
4-42 P.M.	A 2 13/2.0	Test 1/1 —32 seconds
	B 2 13/1.5	Test 1/1 —45 seconds
4:50 P.M.		
Control 3	10/1.0	No test
4:55 P.M.	A 3 13/2.0	Test 1/1 — 7 seconds
	B 3 13/2.0	Test 1/1 —11 seconds
5:05 P.M.		
Control 4	10/1.0	Test 1/1 —50 seconds
5:08 P.M.	A 4 12/2.0	Test 1/1 negative
5:13 P.M.	B 4 12/1.5	Test 1/1 — 9 seconds
Later	A 5 about the same	Test 1/1 —negative
	B 5 as above	Test 1/1 — 8 seconds

is no doubt, therefore, that in eggs with membranes formed by butyric acid the agglutinating substance is fixed, as it is in fertilized eggs. Presumably, the same would hold true for other parthenogenetic methods which render the eggs incapable of fertilization.

The next day the washings were continued. On the sixth, washing *A* again showed a trace of the agglutinating substance, but came negative again on the remaining three washings. The reappearance is to be attributed to the few eggs without membranes which had been active all night, accumulating in this time sufficient agglutinating substance to show in the test though not producing sufficient to show in repeated washings. The *B* eggs came negative in the ninth washing, while the control eggs still gave a 40-second reaction undiluted.

The *B* eggs although exposed longer to the butyric acid did not form membranes, and they continued to produce fertilizin longer than the *A* eggs but in much less quantity than the control eggs. It might therefore, be expected that they would retain capacity for fertilization longer. The fertilization capacities were not

worked out in this experiment; l
his experiments on superposition o
that eggs overexposed to butyric
incompletely.

Ninety-five per cent of the *A* eg
ysis on the next day, beyond t
were intact and healthy looking.
fragmented, or segmented, into a
membrane and appeared cytolyze
tion had disappeared, and there
of dividing eggs as in *A*, but in al
were breaking apart.

A similar experiment on Augus
disappearance of the agglutinati
formation by butyric acid.

Summarizing this section, the
rendered incapable of fertilizatio
the sperm-agglutinating substanc
lost, as in some eggs after repea
previous fertilization, or (c) fixed
The conclusion is therefore justifi
stance is necessary for fertilization

8. SUMMARY OF PART II: SPERMOP

After considering a method for
fertilizin produced by ova of Arba
unfertilized eggs in sea-water produ
large quantities. The secretion be
with breaking of the germinal vesi
urated with it and liberates it by s
without jelly continue to secrete if
repeated washings, or as long as
spermatozoa of Arbacia possess ext
and bind it in definite amounts. I
the binding is evidenced by revers
matozoa. But a stale sperm suspen

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zation by opening the animal and removing the ovaries so that the eggs are contaminated by blood or tissue secretions, and it is evident that the latter in some way inhibit the fertilization reaction, though they could hardly be supposed to have any direct injurious effect upon either sex element.

The necessity of washing the eggs carefully before fertilization, had been forced upon my attention by the poor results of certain experiments; and after some of the results recorded in the first part of the paper had been obtained, the phenomenon appeared to possess great significance, because the failure to fertilize in the case of unwashed eggs was clearly due to the presence of blood of the species. Some element of the blood must block the mechanism of fertilization, and it was clear that the analysis of the phenomenon must aid in understanding the mechanism.

The first experiments were undertaken to ascertain the extent of the inhibiting action of the blood of the sea-urchin. The nature of the effect may be seen from the protocol of the following experiment:

July 7, 1913: The blood of several males and females was obtained by cutting open the shell near the oral membrane and pouring out the perivisceral fluid (blood) in a finger-bowl. After the usual loose clot was formed the plasma was filtered through a soft filter-paper. The eggs used in the experiment came from one female and were washed in sea-water. The experiment was then set up as follows:

1 Quantity of eggs x plus 25 cc. sea-water plus 4 drops 1 per cent sperm
2 Quantity of eggs x plus 12.5 cc. filtered blood plus 12.5 cc. sea-water plus 4 drops 1 per cent sperm
3 Quantity of eggs 2/5 X plus 10 cc. filtered blood plus 4 drops 1 per cent sperm

The fertilizations were made about the same time and the result was that in 1 practically all divided, in 2 only a fraction of 1 per cent divided, in 3 none divided. Moreover in 2 and 3 no fertilization membranes were formed in the undivided eggs and the egg nucleus remained intact; no spindles were formed. And this in spite of the fact that the spermatozoa were very active, so abundant that they formed 'halos' around the eggs by penetrating the jelly, and many reached the surfaces of the eggs.

This experiment was followed by a considerable number carried out in more detail. Four of these experiments are tabulated in table 8. The left-hand column gives the percentage of blood in

the sea-water in which the fertilizations were made. The other vertical columns give the percentages of eggs segmented for each concentration of blood on the same horizontal level. The last reading in each column gives the control, i.e., fertilizations made in sea-water alone with the same amounts of the same eggs and sperm used in the blood solutions. The eggs in the blood solutions were washed in the same way as the controls prior to insemination.

TABLE 8

	JULY 8: MIXED BLOOD OF MALES AND FEMALES	JULY 15: BLOOD OF MALES ALONE		JULY 22: BLOOD OF MALES ALONE
Blood serum in sea-water	Segmented eggs			
		Exp. 1	Exp. 2	
1 per cent	75 per cent	90 per cent	90 per cent	89 per cent
3 per cent		25 per cent	60 per cent	21 per cent
5 per cent	10 per cent	10 per cent	30 per cent	0
7 per cent		1 per cent	30 per cent	6 per cent
9 per cent		25 per cent	45 per cent	19 per cent
10 per cent	about 0.2 per cent	50 per cent		
11 per cent			20 per cent	0
13 per cent			20 per cent	0.1 per cent
15 per cent			1.5 per cent	0
20 per cent	about 0.2 per cent			1.75 per cent
40 per cent	about 0.2 per cent			
100 per cent				0
Control: same eggs in sea-water.....	75 per cent	99 per cent	95 per cent	99 per cent

It will be noticed that in all these cases there is a marked reduction in the percentage of fertilizations when 5 per cent blood is present in the sea-water, greater in some cases than in others, which leads to the conclusion that the inhibitor is present in higher concentrations in some samples of blood than in others. This relation is dealt with below. In Experiments 1 and 2 of July 15, and in the experiment of July 22, there will be noticed after the first fall with increasing blood-concentration a rise and second fall in the percentages of fertilizations as measured by cleavage of the eggs. This is unquestionably of some significance, but I have been unable to ascertain what its meaning may be. The same phenomenon occurred in later experiments.

While the experiments cited give effects only of mixed blood from the two sexes, and the blood of the males alone, other experiments, not so complete in all details, showed that females contain the inhibitor in their blood at least to the same degree as the males. If there is any sexual difference it is in favor of the females. The blood of the males was used in many tests alone because there was danger, in opening the females, of wounding the ovaries and so getting fertilizin into the blood, which as shown beyond, greatly modifies the results. There was of course the danger in using blood from males of getting spermatozoa from wounded testes, but as the eggs were in any case to be fertilized the result could hardly be affected by this; some control experiments showed that this was a rare occurrence in any event.

Individual variability in the amount of the inhibitor present in the blood being indicated in these experiments, a special study was made of this factor in order to test its extent, and if possible to correlate it with the condition of the animals. The individual tests were as follows (table 9): the columns give the percentage of eggs segmented after 2 to 3 hours. All the fertilizations of July 28 were made with a single sperm suspension and a single lot of eggs; similarly for each succeeding date. The various numbers therefore act as controls on one another. In all, a great excess of sperm was used. The counts were carefully made and checked by my assistant, Miss Brockett.

These tests of bloods of individuals bring out very extreme individual differences in the content of fertilization inhibitor in the blood, ranging in fact all the way from no inhibition to absolute inhibition of fertilization. But it is not easy to correlate the variations with the conditions of the individuals. The general hypothesis that I have been led to adopt, provisionally at least, is that the quantity of the inhibitor in any individual blood is related to the size and degree of maturity of the gonads. It would seem to be a very simple matter to determine whether this is or is not the case. However, in the first place, after the beginning of the season there are practically no animals with immature gonads to test, and in the second place, if the hypothesis were correct, it would be impossible to know in advance how long the

TABLE 9

	MALES—20% BLOOD		FEMALES—20% BLOOD	FEMALES—100% BLOOD
July 28				
1	65 per cent	1	75 per cent	11 per cent
2	69 per cent	2	93 per cent	84 per cent
3	71 per cent	3	72 per cent	11 per cent
4	72 per cent	4	45 per cent	1 per cent
5	85 per cent	5	85 per cent	84 per cent
6	82 per cent	6	87 per cent	63 per cent
7	27 per cent	7	89 per cent	81 per cent
8	84 per cent	8	52 per cent	0
		9	80 per cent	24 per cent
		10	92 per cent	93 per cent
		11	6 per cent	2 per cent
		12		55 per cent
		13		0
		14		most
		15		over 90 per cent (rough estimate)

	MALES—100% BLOOD		FEMALES—100% BLOOD
July 29			
9	100 per cent	16	77 per cent
10	85 per cent	17	39 per cent
11	56 per cent	18	100 per cent
12	87 per cent	19	0
13	43 per cent	20	100 per cent
Control 97 % (fertilized in sea-water)			
July 30			
14	2 per cent	21	35 per cent
15	25 per cent	22	100 per cent
16	29 per cent	23	15 per cent
17	93 per cent	24	65 per cent
18	93 per cent	25	19 per cent
19	100 per cent	26	99 per cent
		27	100 per cent
Control 100 per cent (fertilized in sea-water)			
July 31			
20	96 per cent	28	0
21	2 per cent	29	1 per cent
22	74 per cent	30	0
		31	38 per cent
		32	1 per cent
		33	41 per cent
		34	41 per cent
Control 94.5% (fertilized in sea-water)		35	89 per cent
		36	34 per cent

inhibitor might remain in the blood after the gonads were emptied. Thus the mere fact that an individual with small gonads had much inhibitor in its blood would not prove that the gonads had little to do with the formation of the inhibitor, for there would be no way of telling whether the reduction of the gonads was recent or not.

The considerations that led to the adoption of the above hypothesis are (1) that the condition of the gonads is the most variable thing in the summer sea-urchins, which coincides well with the variability of the amount of the inhibitor; (2) in general, the fluctuation through the season of periods of maturity of the sea-urchins coincides very well with the fluctuations in the amount of the inhibitor. Thus the animals were in very fine breeding condition during the first three weeks of July when the first tests showing abundance of the inhibitor in the blood, were made. They were in very poor condition then for about 10 days up to about August 2, when the individual tests were made showing such surprising decrease of the inhibitor. On August 6 when the material became better again, the inhibiting effect of the blood was stronger once more. (3) Although it was difficult, as said, in the individual tests to correlate the amount of the inhibitor with the condition of the animals, yet especially in the case of the females, those with large gonads appeared to have a large amount of inhibitor present. Thus females 28, 29, 30 and 32 were all ripe females that shed eggs when inverted after opening, in a watch crystal, whereas females 31, 33, 34, 35 and 36 were all individuals with medium size ovaries that shed no eggs when similarly inverted. Female 8, on the other hand, had small ovaries, shed no eggs, and yet possessed much inhibitor in its blood.

The conditions that determine the amount of inhibitor in the blood are thus a matter for more careful investigation. It may be possible to regulate the quantity experimentally by injection of fertilizin or of entire eggs. This merely indicates a possible method of approaching the problem.

I spent a great deal of time in endeavoring to meet the possible objection that the inhibiting action of the blood might be due to

contamination with the poisonous secretions of the epidermis, especially of pedicellariae. It seemed at first that this objection might be valid, for the period of my tests coincided with the season of poor material when the blood was relatively free from inhibitor. During this period, instead of following my earlier practice of opening the animal rapidly and pouring out the blood, I very carefully cut within the leathery peristome, avoiding all possible contamination with epidermal secretions and removed the blood with pipettes. This blood showed as a matter of fact little inhibiting effect. However, with the return of animals in good breeding condition, it was easy to show that the inhibitor was contained in the blood itself; and not only this, but also that epidermal secretions so far from increasing the inhibiting action of the blood, actually decreased it, a matter to which we return beyond.

The next question was whether the inhibitor acted on the egg alone, on the sperm alone, or merely by intervening in the reaction between the two? In the experiments undertaken to answer this question it was possible to show that the inhibitor certainly does not act strongly on either the egg or the spermatozoa alone. It is possible to collect eggs or sperm in filtered blood known to have a strong inhibiting action, and, after considerable exposure, to restore by washing nearly full fertilizing power, at least, to both kinds of sexual elements.

July 8: The blood used in this experiment allowed only 1 per cent fertilization when diluted to 5 per cent with sea-water; it therefore contained a great deal of the inhibitor.

1. As regards the effect on eggs alone: 9:41 A.M. The eggs of one female were placed direct from the ovary in some of this filtered blood. Transfers of two drops of eggs to 10 cc. of sea-water plus one drop 1 per cent sperm were made as follows: a. 9:42, b. 9:50, c. 9:55, d. 10:03, e. 10:23. About 60 per cent of each lot segmented. The blood carried over would be about 0.625 per cent. Thus the inhibiting effect was removed to a very great extent by washing. 2. In the case of sperm collected in the same blood and used for fertilization at 1 minute, 12 minutes, 22 minutes, and 37 minutes, no indication of inhibition of fertilizating power as compared with an identical series of controls was observed.

July 17: After 5 minutes' exposure to male blood sperm fertilized 20 per cent of the eggs; control sperm 90 per cent. (sperm = 0.004 per cent).

July 18: Sperm suspensions in two lots of blood, (a) from males, (b) from females. (a) Fertilized 40 per cent in 5 minutes and 25 per cent in 18 minutes, (b) fertilized 30 per cent in 5 minutes and 20 per cent in 18 minutes. Control sperm fertilized 70 per cent each time. (Sperm = 0.0026 per cent)

July 19: Sperm suspensions in two lots of blood, (a) from males, (b) from females. (a) Fertilized 14 per cent in 10 minutes and 18 per cent in 30 minutes; (b) fertilized 23 per cent in 10 minutes and 20 per cent in 30 minutes. Control sperm fertilized 86 per cent in 10 minutes and 76 per cent in 30 minutes. (Sperm = 0.012 per cent).

July 21: Sperm suspensions in blood of males. In this case the sperm fertilized perfectly in two fertilizations. Sperm = 0.007 per cent in first and 0.0068 per cent in second. The blood contained a large amount of inhibitor as only 0.5 per cent segmented in it with 0.6 per cent sperm suspension.

August 1: A very detailed experiment showed that a specimen of blood very powerful in inhibitor had but little effect on the fertilizing power either of eggs or of sperm after washing.

On the whole we must conclude that though exposure to blood containing inhibitor may decrease the fertilizing power of sperm or of eggs, its effect is secondary and can probably be entirely removed by sufficient washing or overcome by sufficient concentration of sperm.

2. MODE OF ACTION OF THE INHIBITOR

It was natural to suppose that the inhibitor acted by occupying the side-chain of the fertilizin with which the sperm unites normally. If this were the case it must be possible to neutralize the agglutinating action of the fertilizin by a sufficient quantity of blood containing the inhibitor. Therefore, if we took two equal amounts of an agglutinating solution and diluted the one with sea-water and the other with blood containing the inhibitor, the agglutinating action ought to disappear more rapidly in the latter than in the former. This, however, is not the case, as the following experiment will show.

July 11, 1913: A given agglutinating solution was tested in the usual way by diluting with sea-water and found to be of 400 agglutinating power. Part of it was then diluted to 1/100 with blood; on test with sea-water sperm suspension it gave two tests of 22 seconds each. Diluted to 1/400 with blood, it gave a 10 second reaction with a sea-water sperm suspension, and an 11 second reaction with a blood sperm suspension.

The same result was obtained in other experiments and I could find no evidence that blood decreased the delicacy of the agglutinating reaction.

If then the fertilizin is essential for fertilization, and if the inhibitor in the blood does not operate by preventing union of this substance with the sperm, it follows that another side-chain of the fertilizin capable of being occupied by the inhibitor is also operative in fertilization. There must be action of the fertilizin on the egg as well as on the sperm, and hence we may assume an ovophile as well as a spermophile side-chain of the fertilizin, and the inhibitor must act by occupying the former.

Now Robertson ('12) has stated that various proteins when added to the sea-water can be shown to inhibit membrane formation by sperm, though as a matter of fact his experiments demonstrate such a result only for ovomucoid in concentrations above 0.25 per cent. It might therefore be supposed that the inhibiting effect of blood of the same species was only a special case of protein inhibition. There are, however, considerations that show such a view to be untenable. (1) As already shown the degree of inhibition by undiluted blood ranges all the way from zero to 100 per cent; and we cannot suppose that the amount of protein in the blood of different individuals varies to the extent required to explain such an extraordinary difference. (2) In the second place the mode of neutralizing the inhibitor, as shown below, involves an enormous increase of colloid content of the blood, for it consists in saturating the blood with fertilizin and dissolved jelly; the blood may thus become actually syrupy in consistency and yet have no inhibiting effect on fertilization. (3) I have tested the effect of egg-albumen in the sea-water up to 0.15 per cent without finding any inhibition of fertilization. Of course in higher concentrations a certain degree of inhibition may occur. However the reasons given under 1 and 2 above, certainly exclude the idea that we are dealing with an effect common to colloids in general, which must be assumed to operate through changes in the membrane. It is necessary to assume some specific substance acting as inhibitor.

Fortunately the idea that the inhibitor operates by occupying an ovophilė side-chain of the fertilizin is capable of a ready test, for then it should be possible to neutralize the inhibiting effect of the blood, which now becomes ex. hyp. a deviation effect, by treating the inhibiting blood with free fertilizin. This substance should bind all molecules of the inhibitor so that they could no longer exert any deviating effect, their bonds for fertilizin being already occupied. This experiment succeeds perfectly as a matter of fact.

Before citing the experiments in detail, I would like to dwell a little more on their significance. In the first place a negative result would go far to overthrow the entire theory, for then it would become necessary to assume, if the theory were to be retained, that the combination of fertilizin and inhibitor could take place in the egg but not in the test tube, an exceedingly improbable assumption. The positive result, actually obtained, is in accord with the theory, and it is exceedingly difficult to frame any other interpretation of such a result. The alternative point of view would be to postulate a neutralization of a hypothetical membrane effect of the inhibitor, a conception exceedingly difficult to support as we have seen, and now rendered doubly difficult through the neutralization of such effect by excess of a substance which by itself in high concentrations actually reduces the percentage of fertilizations. Such an alternative interpretation appears to me impossible.

The experiments

July 21, 1913: The following substances were prepared: (1) Fresh filtered blood of male sea-urchins. (2) Filtrate from 15 cc. sea-water plus 7.5 cc. ripe Arbacia eggs, i.e., an agglutinating solution in sea-water, which gave an 8 second reaction with fresh sperm at 1/1600 dilution. (3) Filtrate from 15 cc. of the same male blood as in 1, plus 7.5 cc. eggs; i.e., part of the blood (1) saturated with the egg secretions. It gave a 10 second agglutinating test at 1/1600 dilution.

Two drops of fresh sperm (50 per cent) were then stirred into 5 cc. of each of the above and into (4) 5 cc. sea-water for control. These sperm suspensions prepared at 10:50 A.M. were thus about 0.6 per cent. One drop of double washed eggs was then added to each sperm suspension 1–4 at 11:45.

TABLE 10

		SEGMENTED EGGS
(1)	0.6 per cent sperm suspension in blood	0.5 per cent
(2)	0.6 per cent sperm suspension in 1600 agglut. power sea-water	99.0 + per cent
(3)	0.6 per cent sperm suspension in 1600 agglut. power blood	100.0 per cent
(4)	0.6 per cent sperm suspension in sea-water (control)	100.0 per cent

This experiment was unusually successful; the eggs and sperm were perfect, giving 100 per cent segmentations (4). The blood contained so much inhibitor that only 0.5 per cent of the eggs segmented (1). But its action was entirely neutralized by the addition of the agglutinating substance (3). Fertilization 2 shows that in the presence of such an excess of sperm, the agglutinating substance itself has but little inhibiting power.

In a similar experiment of July 19, 0.9 per cent sperm suspensions were made in solutions 1 to 6 below, and a drop of eggs added to each with results noted in table 11.

TABLE 11

	FERTILIZATIONS MADE IN	PERCENTAGE OF SEGMENTED EGGS
(1)	Filtered blood of male sea-urchins	0
(2)	Filtered blood of female sea-urchins	0
(3)	800 power agglutinating solution in sea-water	93%
(4)	1600 power agglutinating solution in mixed blood of males and females	73%
(5)	Mixture of equal parts of 1 and 3	0
(6)	50% dilution of 1 with sea-water	0
(7)	Sea-water control	97%

1, 2 and 6 show the inhibiting power of the blood on fertilization even with such enormous excess of sperm; 4 shows neutralization of the inhibiting power by a large quantity of the agglutinating substance although the neutralization was apparently not complete (cf. control); 5 shows that the agglutinating substance contained in an equal part of 800 power sea-water solution was inadequate to neutralize the inhibitor in this sample of blood; a considerable part of the inhibitor must have remained free; it must be present therefore, in high concentration.

Experiments were then made to test the neutralization of the inhibiting power of the blood at various dilutions.

July 22: Used filtered male blood which permitted only 0.5 per cent fertilization. Part of this was saturated with agglutinating substance by addition of 50 per cent by bulk of eggs, and filtered. Fertilizations were then made in a series of sea-water dilutions of each, using a single lot of eggs and of sperm (table 12).

TABLE 12

A Series—Blood alone			B Series—Blood saturated with agglutinating substance about 6400 strong		
	Fertilisations in 0.1% sperm suspensions in	Per cent segmented		Fertilisations in 0.1% sperm suspensions in	Per cent segmented
(1)	1% blood	89.0%	(1)	1%	99.0%
(2)	3%	21.0%	(2)	3%	89.5%
(3)	5%	0	(3)	5%	97.0%
(4)	7%	6.0%	(4)	7%	94.5%
(5)	9%	19.0%	(5)	9%	93.5%
(6)	11%	0	(6)	11%	94.0%
(7)	13%	0.1%	(7)	13%	87.5%
(8)	15%	0	(8)	15%	91.0%
(9)	20%	1.75%	(9)	20%	71.0%
(10)	100%	0	(10)	100%	3.0%
	Control in sea-water	99%			

It will be seen that this sample of blood is very powerful in inhibitor (*A* series), and that the agglutinating substance protects from its action at every concentration. The decreases in percentage of segmented eggs in B9 and B10 are to be attributed probably to excess of the agglutinating substance, which was exceedingly concentrated, and which always has some diminishing effect on percentage of fertilizations when the sperm suspension is not too concentrated. In this experiment it will be noted that the agglutinating substance was 6400 strong as contrasted with 1600 on July 21, and the sperm suspension was six times more dilute.

July 25: (A) Used filtered blood of females; (B) part of *A* saturated with agglutinating substance by addition of eggs; tested 3200 strong. Fertilizations were made in 0.2 per cent sperm suspensions in the following dilutions of each, with per cent of segmented eggs as noted, (table 13):

TABLE 13

A Series			B Series		
	Dilutions	Percentage of segmented eggs		Dilutions	Percentage of segmented eggs
(1)	10%	17%	(1)	10%	73.5%
(2)	40%	5%	(2)	40%	58%
(3)	70%	0	(3)	70%	40%
(4)	100%	0	(4)	100%	6%
(5)	Control in sea-water	58.5%			

The same protective action of the agglutinating substance is shown, with some decrease at the higher concentrations due, presumably, to excess of the agglutinating substance. The fertilization *B*-1 actually is better than the control.

August 6: This experiment was made up of a series of combinations of blood *A* plus fertilizin-saturated blood *B*. Fertilizations made in 0.2 per cent sperm suspensions in each combination.

TABLE 14

(A) Filtered serum of males and females

(B) 23 cc. A plus 11.5 cc. ripe eggs. Centrifuged after 10 min. and filtered; sperm-agglutination test, 1/3200=9 sec.

	Fertilizations in	Percentages of segmentation; 400 of each counted, 200 by Miss Brocket, 200 by self
	5.0 cc. A + 0 B	
(1)	4.9 cc. A + 0.1 cc. B	0.5%
(2)	4.8 cc. A + 0.2 cc. B	0.5%
(3)	4.7 cc. A + 0.3 cc. B	1.75%
(4)	4.6 cc. A + 0.4 cc. B	8.5%
(5)	4.5 cc. A + 0.5 cc. B	25.0%
(6)	4.4 cc. A + 0.6 cc. B	32.75%
(7)	4.3 cc. A + 0.7 cc. B	31.75%
(8)	4.2 cc. A + 0.8 cc. B	72.0%
(9)	4.1 cc. A + 0.9 cc. B	62.25%
(10)	4.0 cc. A + 1.0 cc. B	85.0%
(11)	3.0 cc. A + 2.0 cc. B	82.75%
(12)	2.0 cc. A + 3.0 cc. B	80.5%
(13)	0 A + 5.0 cc. B	50.0%
(14)		30.25%
Control in sea-water		89.5%

The protective action of the agglutinating substance gradually rises up to number 10 and then falls off, owing, presumably, to excess of agglutinating substance.

I have given a considerable number of the experiments because the matter under consideration is of great significance. There can be no question that some substance derived from the eggs themselves protects against the inhibiting substance in the blood. Until some method is devised for obtaining the agglutinating substance pure, it is impossible to meet entirely the objection that it may be some substance other than the agglutinating substance that protects against the inhibitor. The agglutinating substance can, however, be demonstrated in high concentrations, as the protocols of the experiments show. Until some reason for a contrary assumption is shown, the neutralization of the inhibitor

must be attributed to this, which is indeed the only assumption consistent with the other known facts.

Now if the agglutinating substance protects from the inhibitor by occupying its combining group, it would be logical to expect to find in some tissue of the species another kind of molecule possessing an identical side-chain and hence equally capable of protecting against the inhibitor. Such a substance was found in the shell but not in the intestine. A sample experiment may be cited.

August 2, 1913: A large quantity of blood was collected from females, and the serum filtered. (A) One part set aside. (B) A second part was saturated with the agglutinating substance by addition of eggs; *B* tested to 1/3200; (C) To a third part was added a large quantity of the shell and peristomes of the sea-urchins used: *B* and *C* filtered again. Fertilizations were then made in 0.2 per cent sperm suspensions in identical concentrations of each in sea-water as shown in table 15.

TABLE 15

	DILUTIONS OF SERA	PERCENTAGES OF EGGS SEGMENTED		
		A	B	C
(1)	1%	98.0%	98%	97%
(2)	10%	94.0%	99+%	96%
(3)	50%	58.5%	96%	99%
(4)	100%	15.0%	94%	97%
Control fertilized in		sea-water 99%		

The conclusion would seem to be that shell secretions (poison from pedicellariae?) protect against the inhibitor in the blood. The inhibitor was not very strong in this case, and test of *C* showed that it contained a small quantity of agglutinating substance owing, as sometimes happened, to injuries to the ovaries in collecting the blood, but there did not seem to be enough (7 second reaction undiluted) to influence the result.

The above result was obtained in an attempt to meet the possible objection that the inhibiting action of the blood might be due to the inclusion of poison from pedicellariae. It appears, on the contrary, that shell-secretions decrease the inhibiting action of the blood, an entirely unexpected result.

As the result met the anticipated objection and did not appear to lie in the main line of the research, the experiment was performed only three times in all, with essentially similar results.

To explain such a result one of two hypotheses is possible: (1) that shell secretions destroy the inhibitor; (2) that they unite with its inhibiting side-chain, and thus possess a combining group identical with the ovophile group of the fertilizin; though they do not possess the spermophile group. The reaction takes place rapidly; hence the second assumption is more likely.

3. SUMMARY OF PART III

In this section we have shown:

1. That the blood of male and female sea-urchins contains a varying amount of a substance which inhibits fertilization without exerting any injurious effect upon either the sperm or the ova. This substance is probably more abundant in the blood of mature individuals than in others.

2. The inhibitor does not act by blocking the combination of the sperm receptors with the fertilizin, for it has no inhibiting effect upon the agglutinating action of the fertilizin.

3. It acts by occupying the ovophile group of the fertilizin, thus preventing action of the latter upon the egg by union with egg receptors.

4. This conclusion is demonstrated by the fact that the inhibitor may be entirely neutralized by a sufficient quantity of the agglutinating substance.

IV. GENERAL DISCUSSION

The essential conception that results from the experiments is that the formation of the fertilization membrane is due to activation of an ovogenous substance, which I have named fertilizin to express this idea. Following insemination any excess of the fertilizin is bound, or neutralized, by another ovogenous substance, which I have named anti-fertilizin, and polyspermy is thereby prevented. The experiments demonstrate that reactions in fertilization occur as described in sections II and III, but the

intimate nature of the reactions themselves is unknown. To represent them in terms of the Ehrlich hypothesis as definite lock-and-key chemical combinations is of course to go beyond the facts. But it is necessary in some way to gain definiteness of formulation not only for purposes of description, but also to obtain a definite working hypothesis. The method of formulation which I have adopted has both of these advantages. Indeed some of the experiments could hardly have been suggested by any other hypothesis.

1. THE MECHANISM OF FERTILIZATION

In terms of such an hypothesis the mechanism of fertilization may be summed up in a diagram (fig. 1, sector 1). The cortex of the egg contains the fertilizin (see explanation of symbols) of which three molecules are represented each with a spermophile

Fig. 1 In successive sectors of the egg there are represented the mechanism of fertilization and the blocks to the mechanism, as follows:

Sector 1 The arrangement of substances in the unfertilized egg and in the spermatozoon that are active in fertilization. See explanation of symbols.

Sector 2 The mechanism of normal fertilization. The sperm receptor unites with the spermophile group of the fertilizin and the egg-receptors with the ovophile group of the fertilizin owing to activation of the latter by the sperm (a). Molecules of the anti-fertilizin combine with the spermophile group of the adjacent fertilizin (b and c) and thus block the way for supernumerary spermatozoa. This is the postulated mechanism for prevention of polyspermy. At the same time molecules b and c of the fertilizin have also united with the egg-receptors.

Sector 3 Inhibition of fertilization by loss of the active body, fertilizin.

Sector 4 Theory of antagonistic action of spermatozoa of different phyla. The sperm receptors are occupied by combining groups cast off by the antagonistic spermatozoa.

Sector 5 Fertilization is blocked by occupancy of the egg-receptors. Purely hypothetical.

Sector 6 Theory of inhibitory action of blood of the same species. The ovophile group of the fertilizin is occupied by molecules in the blood (inhibitor) possessing the same combining group as the egg-receptors. Molecules of the blood inhibitor also shown in the medium.

The fertilizin is represented in the diagram as occurring only in the cortex of the egg. But it also occurs in high concentration in the jelly surrounding the egg. The spermatozoon must thus normally arrive at the egg-membrane loaded with combined fertilizin. This fact, however, makes no essential difference in the theory, and its representation would complicate the diagram.

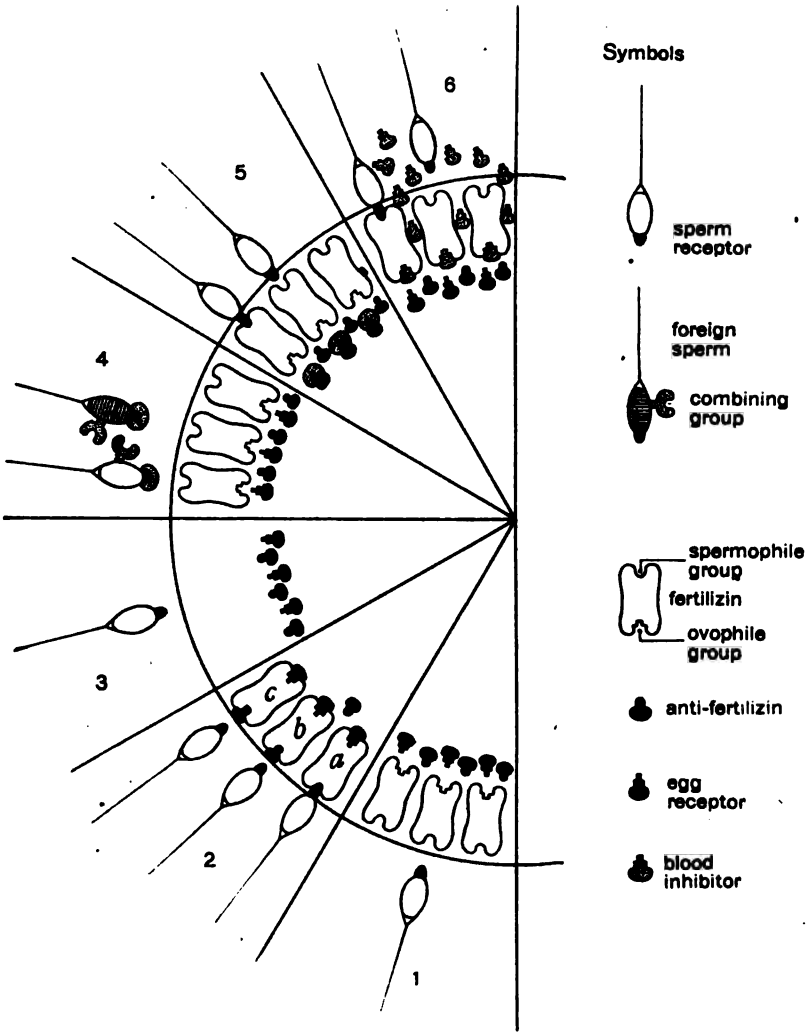
Symbols
sperm receptor
foreign sperm
combining group
spermophile group
fertilizin
ovophile group
anti-fertilizin
egg receptor
blood inhibitor
1
2
3
4
5
6
a
b
c

Figure 1

and an ovophile combining group. The spermatozoon is represented with a receptor capable of fitting the spermophile group of the fertilizin; the egg similarly contains receptors capable of fitting the ovophile group of the fertilizin. Within the egg represented as alternating with the egg-receptors, are also found certain molecules (anti-fertilizin) capable of uniting with the spermophile group of the fertilizin.

Union of a sperm receptor with a spermophile group of the fertilizin molecule activates the latter so that the ovophile group forms a union with an egg-receptor (fig. 1, sector 2, *a*); and I have postulated that the fertilizing action is the result of this union. But the activation of the fertilizin can by no means be regarded as confined to those molecules bound by the single penetrating spermatozoon. On the contrary, as we have seen that all the spermatozoa used in insemination bind only a minute proportion of the fertilizin contained in the eggs, it is necessary to assume that the activation of the fertilizin spreads beyond the point of attack of the successful spermatozoon; this is represented in figure 1, sector 2, *b* and *c*. Inasmuch as membrane formation is practically instantaneous all around the cortex, the activation of the fertilizin must spread with extreme rapidity; an activated molecule of the fertilizin must transmit the activated condition to its neighbor, in a fashion that may be compared by analogy to the spread of a stimulus. The assumption here is no different in principle from that which must be made in any theory of the fertilizing effect of the spermatozoon, and therefore, no new difficulty is introduced.

The postulated mechanism admits of five blocks in the process of fertilization viz.: 1. Absence of the fertilizin (fig. 1, sector 3); 2. Occupancy of the sperm receptors (fig. 1, sector 4); 3. Occupancy of the egg receptors (fig. 1, sector 5); 4. Occupancy of the ovophile side-chain of the fertilizin (fig. 1, sector 6); 5. Occupancy of the spermophile side-chain (fig. 1, sector 2, *b* and *c*). Of these we have examined and discussed in the body of this paper the first, fourth, and fifth, the first in the case of the long washed eggs, (p. 549) the fourth in the case of the inhibitor contained in the blood (p. 563), and the fifth in the case of the non-fertilizable

condition of the already fertilized eggs or those with membranes formed by other methods (p. 556). These cases have been sufficiently discussed already.

But there are two methods of blocking the mechanism according to the hypothesis, which we have not found, viz.: 2 (supra) the occupancy of the sperm receptors in such a way that these side-chains are no longer free to unite with the spermophile group of the fertilizin, which would result in loss of fertilizing power of the spermatozoon (fig. 1, sector 4); and 3 (supra), viz.: occupancy of the egg receptors, or molecules of the protoplasm with which the fertilizin reacts (fig. 1, sector 5). The discovery of these missing links in the theory would serve as strong confirmatory evidence of its essential correctness.

The latter of these two missing links remains purely hypothetical, but I think it is possible that the former may be represented by the phenomenon of the antagonism of sperm suspensions of different animal phyla as described first by Godlewski ('10 and '11) and subsequently by Herlant ('12, *a* and *b*).

These two authors give quite different interpretations of the results. While Godlewski, relying on the analogy of the antagonistic action of heterogenous hemolytic sera, regards the phenomenon as proof of Loeb's 'lysin theory' of fertilization, Herlant is more conservative, and believes that the explanation cannot be sought in any fundamental alteration of either sexual element; but is rather to be found in a purely 'humoral mechanism,' (i.e., in the effect of substance accompanying the spermatozoa) which he supposes to modify the physical state of the surface of the eggs in such way that the spermatozoa cannot penetrate. Herlant apparently overlooks the fact that penetration of the spermatozoon is not necessary for the cortical changes, as has been shown both by Loeb ('09 and '13) and myself ('12). There is nothing in the results of either author inconsistent with the idea, that the mechanism of inhibition in this case may be due to what I have called, "occupancy of the sperm-receptors" as indiated in sector 4 of the diagram. This may be taken to mean merely such mutual action of the spermatozoa (or extractives thereof) on each other as to inhibit the union with the fertilizin.

This could readily be tested experimentally, for, in such a case, the mixed sperm suspension should no longer agglutinate on exposure to the fertilizin of the eggs used in the experiment. If, for instance, we were to find that sperm suspensions of Arbacia and Nereis were antagonistic in the sense of Godlewsky, so that mixtures of the two would not fertilize Arbacia eggs, then according to my hypothesis the Arbacia sperm in the inhibited mixture would not agglutinate with Arbacia fertilizin. The experiment yet remains to be performed.

2. THE TWO PHASES OF FERTILIZATION

In the second study of this series (Lillie '11) I showed that fertilization involves two phases in the case of the egg of Nereis; the first phase is accomplished before the penetration of the spermatozoon, and leads to changes in the cortex of the egg analogous to membrane formation in the sea-urchins. If the spermatozoon be then prevented from entering, the egg completes the maturation process only and does not segment. The cleavage of the egg is casued by some action of the spermatozoon after penetration. Loeb had also shown by entirely different methods the existence of two similar phases in the fertilization of the ovum of the sea-urchin.

The question, therefore, arises, does the mechanism of fertilization discussed in this paper deal only with the first, or with both phases of fertilization? To this it must be answered that the mechanism in question is primarily the mechanism of membrane formation. The described results demonstrate this so positively that farther discussion is superfluous on this point.

But the question remains whether there is any relation of such cortical changes to the internal phase of fertilization? Whether, for instance, if the spermatozoon could enter the egg without first forming the combination with the cortical fertilizin, it would then be in condition to carry out the second phase of fertilization, and cause segmentation of the egg? It has been shown, as is well known, that the egg can be induced to develop apparently without the cortical changes involved in membrane formation,

by the use of hypertonic sea-water for instance. It therefore seems possible that the two phases of fertilization may be disconnected in the case of the spermatozoon also, activated perhaps by different parts of it. On the other hand there is the possibility that fertilization is a continuous process and that the second step cannot proceed if the first be skipped; that the spermatozoon in other words receives some necessary modification, by union with the fertilizin or other substances experienced in the cortex of the egg.

The latter point of view seems more probable to me for the following reasons. In the first place I have made some yet unpublished observations in the case of Nereis which show that if the cortical changes be induced by artificial means there is a brief period in which insemination of the eggs may be followed by penetration of the spermatozoon, but without causing cleavage of the egg. Such a result might conceivably be due to a lack of synchrony between the movements of the egg-nucleus and the sperm-nucleus owing to the start given the egg-nucleus by the treatment prior to insemination. This explanation does not seem to me to be entirely reasonable, because, in Nereis, the sperm enters during maturation and has therefore abundance of time to prepare for union with the egg-nucleus. It seems more probable that the egg has lost something which renders the introduced sperm inefficacious. As a matter of fact, all the fertilizin is thrown off or neutralized in Nereis during the cortical changes, as I have determined by numerous experiments. Miss Allyn ('12) also determined for Chaetopterus that potassium chloride, which starts parthenogenetic development in this form, "initiates changes which increase with time and which are inimical to normal fertilization. They do not prevent the entrance of the sperm into the egg, but they prevent the normal behavior of the sperm in the egg." The cortical changes are not very obvious in Chaetopterus, but it is reasonable to suppose by analogy that in initiating parthenogenesis the fertilizin is bound; the sperm entering after this event are relatively inefficacious.

Dr. G. L. Kite kindly allows me to refer to some yet unpublished experiments performed last summer in which he injected,

by means of the Barber apparatus, from two or three to over twenty spermatozoa of the star-fish into eggs of the same species. There was never any indication of cleavage. The experiments were limited owing to interruption of Dr. Kite's work, so that the evidence in this case is not yet wholly conclusive; but the results indicate the necessity for normal penetration, if the spermatozoon is to be effective.

We therefore see a second function of the fertilizin, viz.: to prepare the spermatozoon for exercising fertilization effect in the interior of the egg. *The spermatozoon needs itself to be fertilized.*

De Meyer ('11) has observed swelling of the head of the spermatozoon under the action of egg-extractives, and I have described the same thing for Nereis ('13). But how such changes intervene in the internal fertilization reaction remains problematical. It is improbable that the swelling itself is significant: it is rather to be taken as indication of a more deeply seated physiological change which is essential for the completion of subsequent reactions.

3. THE LYSIN THEORY OF FERTILIZATION

Loeb's lysin theory of fertilization may be taken as the type of all theories which postulate that the spermatozoon produces and bears a fertilizing substance. Loeb holds that the spermatozoon causes membrane formation and thus initiates development by virtue of a lysin, and he holds this idea not because he, or some other, has isolated this substance and tested its action, but because the action of the spermatozoon in initiating development resembles the action of certain cytolytic substances, more especially, fatty acids, certain glucosides and the blood of some foreign species. Indeed he holds that all cytolytic agents induce membrane formation, and the action of the spermatozoon is regarded as merely a special instance. The spermatozoon, accordint to Loeb, introduces a second substance into the egg whose function it is to inhibit the harmful effects of membrane formation. However the effect of fertilization in increasing the rate of oxidation within the egg is due not to a catalyzer introduced by the spermatozoon but to the activation of catalyzers

already in the egg as shown by the fact that the rate of development is not increased by superposing fertilization on parthenogenesis or by polyspermic fertilization.

The evidence for the existence of a fertilizing lysin in the sperm is thus entirely analogical and would apply equally well to the conception that the sperm causes membrane formation by activating a substance in the egg, which would make the first part of Loeb's theory of fertilization consistent with the last part. Direct evidence is lacking for the lysin theory, but the whole series of facts described in this paper is direct evidence for the existence of the fertilizing substance in the ovum. We have obtained it in sea-water solution and we have studied its effects both on the spermatozoon and on the ovum and have found it to answer to all the necessary requirements. If the conclusions are accepted, we can hardly continue to believe in the existence of the hypothetical sperm-carried lysin.

But apart from this consideration, the lysin theory exhibits certain features of inadequacy. In the first place the failure of attempts to isolate such a substance from spermatozoa and obtain fertilization can be explained, as Loeb has pointed out, on the assumption that, though presumably present in sperm extracts, it lacks power of penetration in the isolated condition and needs the penetrative power of the living spermatozoon to carry it through the egg membrane. A similar assumption would have to be made with reference to the sperm receptors in my theory; so that this lacuna is not peculiar to the lysin theory.

In the second place the lysin theory affords no explanation whatever of the non-fertilizable condition of fertilized eggs. If membrane formation is the result of a superficial cytolysis produced directly by a lysin carried by the sperm and checked by operation of a second spermatic substance effective after penetration of the spermatozoon, what is to prevent a second cytolysis by a second insemination? There is nothing in the lysin theory that affords the least explanation of this universal phenomenon; even concentrated sperm suspensions do not refertilize or exert a 'cytolytic' effect on fertilized eggs.

Loeb has noted that it is impossible to ascribe the increase of rate of oxidation after fertilization to the introduction of a cata-

lyzer by the sperm because polyspermy does not increase the rate of development over the normal. But he has neglected to note that the same argument applies to his lysin theory; if this theory were correct we should expect that polyspermy would increase the cortical cytolysis above the normal, and result in an unusually distended fertilization membrane; but this is not the case; membrane formation is not excessive, so far as observed, in polyspermic eggs. Yet the lysin theory requires that this should be the case.

It is also inconceivable that a lysin could act effectively in such high dilutions as the respective bulks of spermatozoon and ovum render necessary. Wilson estimates the bulk of the spermatozoon at about 1/400,000 that of the egg in the sea-urchin. The sperm head is not visibly diminished after entrance so it would be unreasonable to suppose that more than 10 per cent of its substance has been used in membrane formation. The lysin theory supposes that a spermatozoon cytolyzes a bulk of 4,000,000 times its lysin content! Not only so, but inasmuch as it is introduced at one point the lysin must diffuse with a speed and evenness that renders it effective simultaneously, and very quickly, at all points on the surface of the egg. This conception seems to me to be inconceivable, for the theory of lysin action presupposes a union molecule for molecule over the affected surface. We should also have a large spermatozoon for large eggs and a small spermatozoon for small eggs on the basis of a lysin theory, which is not the case.

These considerations are arguments not only against the lysin theory but also against any theory that presupposes that the spermatozoon is the bearer of a substance acting directly in causing formation of the cortical changes of fertilization.[5]

I may be allowed finally to point out that, even if some form of sperm extract should be shown to be effective in the production of the cortical change, such a result would be as consistent with the theory of indirect action or activation of an ovogenous substance as with the theory of direct action and would be no argument against the preceding considerations.

[5] Although Professor Loeb has committed himself very definitely to the lysin theory, he has not failed to note the theoretical possibility of the activation hypothesis (see Loeb 1913, p. 235; earlier reference given here).

4. THE THEORY OF PARTHENOGENESIS

A theory of parthenogenesis must also be a theory of fertilization, at least for the phenomena common to both. Similarly a theory of fertilization must be consistent with the facts of parthenogenesis. This has been generally recognized and it is one of Loeb's great services to have brought about the recognition of this principle and to bring the problem of fertilization definitely into the field of physiology. That the fertilizin theory is consistent in this respect is sufficiently obvious; indeed it makes the action of the sperm itself into a kind of parthenogenesis, for the sperm activates the fertilizing substance already present in the egg. The egg is self-fertilizing.

However, it lies beyond the province of this paper to consider the various methods of artificial parthenogenesis in detail and to examine the question of their consistency with the theory. It is obvious, I think, that the fertilizin theory from its very nature is more elastic with reference to this problem than any other, and is in general, therefore, more consistent with the established fact of the great variety of proved parthenogenetic agents.

Obviously it suggests certain lines of experimentation in parthenogenesis. Glaser has already followed one of these lines with some success, viz.: The effect of exposure of eggs to extracts or secretions of eggs of their own species; and he has ascertained that such extracts are rather efficient parthenogenetic agents in the case of Arbacia, especially when followed by treatment with hypertonic sea-water. He did not, however, observe the formation of membranes in eggs so treated, which leaves the mechanism of action rather obscure; farther analysis of the result is desirable.

But the whole procedure in experiments in parthenogenesis must take on a different aspect as soon as it is realized that the entire process, and not merely part of it, consists in acceleration of possible combinations in the egg, or increase in avidity of certain chemical groups.

It is not possible to separate the problem of fertilization from the general problems of cellular physiology; I may therefore be

allowed to point out the impossibility of regarding the interchange between the egg and its enviroment as controlled exclusively by diffusion through a semi-permeable membrane. However significant the rôle of permeability of the membrane may be, my experiments show that the egg cell is by no means bound in its exchange to diffusible substances; but that the membrane must be regarded as an active factor, and not only a passive one. Twenty years ago diffusion was assumed to play the chief rôle in the absorption from the intestine or in excretion by the kidney epithelium. Today we know that the excretion of urea means work by the kidney cell; it is an active, not a passive, process. Similarly the secretion of fertilizin by the egg cell, and possibly other cortical phenomena, must be regarded as active processes.

The ordinary chemical analysis of the cell begins with destruction of its more highly organized living constituents; it is obvious that such methods are inadequate for the investigation of the immediate reactions in living protoplasm. The results of these experiments may then gain a still broader interest if they may be taken to indicate a method of studying such reactions by the use of living cells as indicators.

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STUDIES OF FERTILIZATION.

VII. ANALYSIS OF VARIATIONS IN THE FERTILIZING POWER OF SPERM SUSPENSIONS OF ARBACIA.

FRANK R. LILLIE.

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I. INTRODUCTION.

In his epoch-making "Expériences pour servir a l'histoire de la génération des animaux et des plantes" published in 1785 the Abbé Spallanzani describes among his numerous experiments on fertilization and artificial parthenogenesis some determinations concerning the minimal quantity of sperm necessary to fertilize the eggs of the frog. He found that he could get perfect fertilization with seminal fluid diluted 2,720 times with water. At greater dilutions the percentage of fertilized eggs began to fall off, but some eggs fertilized up to a dilution of about 20,000 times. He calculated that the weight of the "spermatic particles" necessary to fertilize an egg was 1/2,994,687,500 of a grain, and that the volume of the egg in proportion to the volume of spermatic particles necessary to fertilize it is as 1,064,777,777: 1. In 1824 Prevost et Dumas confirmed these calculations.

So far as I know such experiments have not been repeated. The reason for this would appear to be that when it was once established that only a single spermatozoön unites with each ovum in fertilization all such quantitative studies of the fertilizing power of sperm dilutions appeared to have lost their point. So long as it was assumed (as was generally the case) that the fertilizing power of the spermatozoön is a function of its motility

alone, that is, of its capacity to "penetrate the ovum," there could be no object in quantitative studies. But as it came to be recognized that the fertilizing power of the spermatozoön is associated with some definite substance that it bears, possibly either a lysin (Loeb) or an activator (self), the problem assumes a different aspect; for it is obvious that if the sperm should lose such a substance in any way, its fertilizing power would be lost even though its motility should be preserved unimpaired. In such a case the relative fertilizing power of sperm suspensions could not be measured either in terms of concentration or of activity of the spermatozoa. Variations in the fertilizing power of suspensions of known concentrations might, therefore, be a measure of the loss of the postulated fertilizing substance. On reflection it is obvious that the spermatic substance in question must be loosely bound to the sperm, because it exerts its first effect, that of inducing cortical changes in the egg, before penetration, as I have shown for *Nereis*, and Loeb for certain hybrid combinations; at this time, therefore, the spermatozoön must set free its receptors[1] (activators).

Recently Glaser (1913 and 1915) has maintained that in *Arbacia* more than one spermatozoön is needed for fertilization of the egg, even though only one actually penetrates. The observations on which this conclusion rests are no doubt correct, *under the given conditions*, and I have made similar observations, as will appear in the course of the present paper. But it by no means follows from the observations that a single spermatozoön may not be adequate under other conditions (and this can be demonstrated). We cannot, however, deny *a priori* the possibility that for the initial phases of fertilization a number of spermatozoa may be of assistance though only one enters and is concerned in later phases. If the phenomena of fertilization are to receive a physiological, and ultimately a chemical, inter-

[1] In study VI. (1914), I propounded a theory of fertilization according to which the initiation of development of the egg is due to activation of an ovogenous substance, which I named fertilizin, contained in the cortex of the egg. In fertilization such activation is caused by a certain constituent of the sperm, which I called the sperm receptors; and the action of the fertilizin thus aroused must be on certain substances of the egg which I named in general egg-receptors. From a chemical point of view therefore we must have an interaction of three substances (or groups of substances), viz., sperm receptors, fertilizin, and egg receptors.

pretation, quantitative questions may be of serious significance.

It would seem to be a perfectly simple matter to determine the greatest dilution of sperm at which any fertilization takes place, and to express in the form of a curve, from percentages of eggs fertilized, the rate of loss of fertilizing power due to dilution. This was the very simple problem with which the present investigation began. However the results were in the highest degree contradictory; the same lot of sperm might vary in a period of half an hour from 1/1,024 to 1/9,000,000 (or less) of 1 per cent. dilution in its power to fertilize the same percentage of a single lot of eggs. The investigation, therefore, turned to the problem of such variations and their cause.

II. Experiments.

1. *Methods.*

Quantitative methods cannot possibly be as rigorous in a problem of this kind as in a purely chemical problem. In the first place we have to deal with variable reagents in the ova and sperm of *Arbacia;* and in the second place the initial measurements must be made rather hurriedly, so as to ensure freshness of the reagents, and under conditions that do not injure their vitality; the available quantities of material also limit the methods of measurement.

Sperm.—The standard for measurements of sperm dilutions is the "dry sperm"; *i. e.*, the thick creamy mass that exudes from ripe testes of *Arbacia.* If a ripe male be opened and inverted in a dry Syracuse watch crystal a certain amount entirely free from foreign admixture usually flows from the genital pores and collects in a mass in the crystal. While this may in certain cases be as much as 2 c.c., usually it is a much smaller quantity. It is quite impracticable to measure this by graduated pipettes; I have therefore used a drop of this dry sperm from bulb pipettes of fairly uniform openings as a standard, and, reckoning 30 such drops to the cubic centimeter, have made "1 per cent. sperm suspensions" by the addition of such a drop to 3.3 c.c. of sea water. This is the standard suspension from which most of the experiments proceed, and all sperm suspensions are expressed in fractions of such a 1 per cent. suspension. Given perfectly

dry sperm, the initial variation due to the method cannot be very great in relation to the tremendous range of variation in fertilizing capacity of sperm due to other causes. Indeed it is a vanishing quantity.

Eggs.—Egg concentration is a factor of relatively slight significance within the limits of the experiments, as will appear from the facts to be presented. Within a very wide range it does not affect the result measured in percentage of fertilized eggs. It is measured roughly by allowing washed eggs to settle for half an hour in a 100-c.c. graduated cylinder, and expressing the quantity there settled as a percentage of the entire fluid. There is of course for every concentration of sperm an egg concentration that is above the optimum for percentage of fertilization. But, as will be seen from the tables, such egg concentration lies beyond the concentration used in most experiments.

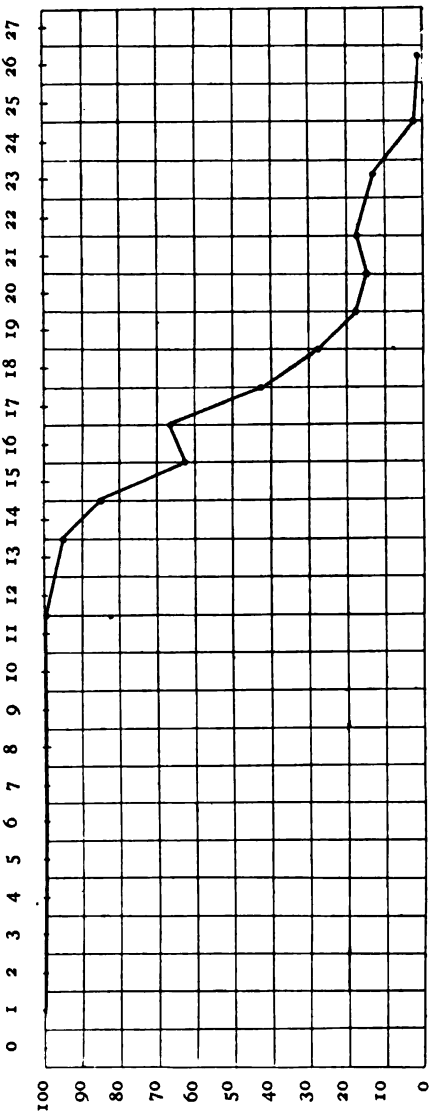
In most cases segmentation of the eggs was used as the criterion of fertilization, but membrane formation was also used in some cases, especially in high concentrations of sperm where many eggs failed to segment owing to polyspermy.

Formulation of Results.—The fertilizing power of sperm suspensions is expressed in curves whose ordinates are percentages of fertilization, and the abscissæ a geometrical series of dilutions of 1 per cent. sperm in powers of 2. This method was adopted for the abscissæ because of the method of successive half dilutions used in many experiments, and because the enormous range of fertilizing power made it impossible to compare results on one scale with an arithmetical progression. When it is realized that the fertilizing power may cease at 1/156 of 1 per cent., or extend to 1/90,000,000 the necessity of the geometrical series in the abscissæ will become apparent.

2. *The Optimum Curve of Dilutions.*

We may begin with the optimum curve of dilutions (Curve 1), because this answers most completely, and probably fully, to the current expectation that a single spermatozoön suffices for the fertilization of an egg. This curve is prepared from data of experiments calculated to bring eggs and sperm together in the freshest possible condition of the sperm. In general measured

quantities of washed eggs were put in measured amounts of sea-water, and measured quantities of definitely calibrated sperm suspensions added and stirred in as uniformly as possible.



CURVE 1.

A control of unfertilized eggs in sea-water was always kept to guard against chance fertilizations. To illustrate: the last four determinations of the curve were made as follows: In four

crystallization dishes were placed 1,000 c.c. sea-water (*A*), 3,000 c.c. sea-water (*B*), 1,000 c.c. sea-water (*C*), 3,000 c.c. sea-water (*D*). To each was added 2 c.c. of a washed egg-suspension (about 3 per cent. to 5 per cent.). The sperm was then prepared as follows: (1) one drop dry sperm to 3.3 c.c. sea-water at 9.43 A.M. = 1 per cent.; (2) 1 c.c. of sperm 1 to 99 c.c. sea-water 9.43.30 A.M. = 1/100 per cent.; (3) 1 c.c. sperm 1 to 999 c.c. sea-water 9.45.30 = 1/1000 per cent. To *A* was added 1 drop sperm 2(1/100 per cent.) at 9.43.45; to *B* one drop sperm 2(1/100 per cent.) 9.44; to *C* one drop sperm 3(1/1000 per cent.) 9.45.45; to *D* one drop sperm 3(1/1000 per cent.) 9.45.45. An assistant stirred in the sperm thoroughly as added. The sperm concentration in *A* was therefore 1/100 × 1/30 × 1/1000 = 1/3,000,000 per cent.; in *B* it was 1/9,000,000 per cent.; in *C* 1/30,000,000 per cent.; in *D* 1/90,000,000 per cent. 1/3,000,000 per cent. falls between 21 and 22 on the scale, and the others as shown. The exact times of mixing the sperm are given because, as will appear beyond, time is an extremely important factor with reference to fertilizing power.

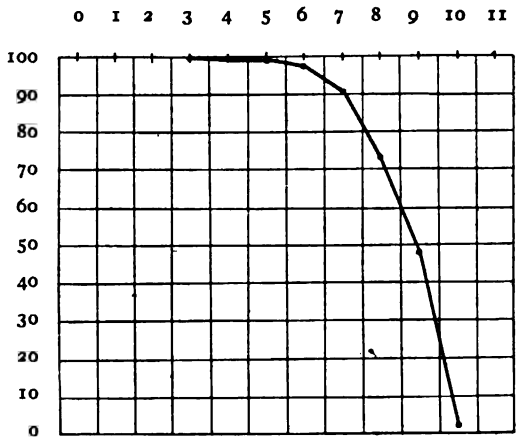
To appreciate the extent of this dilution it may be said that beyond a dilution of 1/10,000 per cent. (between 13 and 14 on the scale) one can rarely find a single spermatozoön in the jelly of the fertilized eggs. At about 1/2000 per cent. (11 on the scale) the sperm suspension does not even appear opalescent. We may therefore feel reasonably sure that beyond about 14 or 15 on the scale a single spermatozoön certainly suffices to completely fertilize an egg.

In further elucidation of the curve I may say that the critical (steep) part was covered by several determinations for each point. Thus there are five determinations averaged for the positions between 13 and 15. Seven between 15 and 18, five between 18 and 20, and six between 20 and 21. The determinations beyond 21 are single determinations. For the first part of the curve up to 13, there are numerous determinations. There are great variations in the single determinations compared with one another; these averages must therefore be regarded only as approximate values. With a sufficiently large number of determinations the irregularities between 15 and 17 and between 19

and 22 would no doubt disappear. But it is improbable that the general form of the curve would undergo any essential change even with a much more extensive series of determinations.

3. *Curves of Successive Half-dilutions.*

In contrast to these results, and for the purpose of defining the character of the main problem sharply, we may next consider the fertilizing power of a series of half dilutions of a 1 per cent. sperm suspension. The curves from these experiments furnish an almost incredible contrast to the one already given; as an example we may examine the following strikingly regular curve, Fig. 2. The first member of this series was a 1/8 per cent.



CURVE 2.

sperm suspension freshly prepared, thus falling in position 3 on the scale; 8 c.c. of this was taken (No. 1); to 4 c.c. of 1, 4 c.c. of sea-water was added (No. 2) = 1/16 per cent., ($1/2^4$); to 4 c.c. of 2, 4 c.c. of sea-water was added (No. 3) = 1/32 per cent., ($1/2^5$); this was continued eight places to $1/2^{10}$. Four drops of a 10 per cent. egg-suspension was then added to each, and the percentage of segmented eggs was counted three hours later. Plotted they give the above curve. In this case it will be seen that the fertilizing power almost ceases at $1/2^{10}$ = 1/1024 per cent. sperm suspension. The eggs and sperm were not at fault

because a parallel control series, in which the same quantities of the same lot of eggs were first placed in the same quantities of sea-water and sufficient of the original 1 per cent. sperm suspension added to make similar sperm dilutions, showed over 95 per cent. cleavage in each case, and actually 99 per cent. in No. 8 of the control where the sperm dilution was 1/1200 per cent. As a further control it may be added that eggs which fail to fertilize in such relatively concentrated sperm suspensions may all be fertilized by the subsequent addition of a trace of perfectly fresh sperm.

The type of experiment just cited was the first undertaken, and for a time it seemed to offer an almost insoluble problem, though the real explanation turned out to be extremely simple. I have twenty curves from similar experiments, fourteen of which run out absolutely from the third to the twelfth place on the scale (*i. e.*, from 1/8 per cent. to 1/4096 per cent.); in the remaining 6 (as in the curve just given) the dilutions were not carried far enough to reach the zero point, but they agree in principle with the others.

A number of control experiments demonstrated the relative lack of significance of the actual sperm concentrations. As one of these I may mention experiment C of August 3. In this case a series of sperm dilutions in powers of 4 was made from 1 per cent. The proportion of eggs fertilized ran off to 1 per cent. at $1/4^5$ ($1/2^{10}$) and to 0 at $1/4^6$ = 1/4096 per cent. But one drop of a 0.1 per cent. suspension of the original 1 per cent. sperm added to eggs in 200 c.c. sea-water fertilized 94 per cent. of them (control for sperm). Thus the control fertilized almost perfectly at 1/60,000 per cent. dilution, whereas the fifth member of the series of dilutions $1/2^{10}$ (1/1024 per cent. sperm) fertilized only 1 per cent. The actual concentration of the sperm is thus not the most significant thing.

This is also brought out strikingly in the following experiment (August 14). A series of half sperm dilutions was made as usual (Series *A*) 2 c.c. in each dish; to a second series (series *B*) of dishes was added 2 c.c. sea-water each and 4 drops of an egg-suspension. The numbers of series *B* were then inseminated by one drop each of sperm from the corresponding number of *A*, thus

diluting the sperm about 1/60. To each of the *A* series (except 1) four drops of the same egg-suspension was then added. The resulting percentages of fertilization are given in Table I.

TABLE I.

A.		*B.*	
1. (1%)	–	1. (1/60%)	– 99%
2. (1/2%)	– 96.5%	2. (1/120%)	– 99.5%
3. (1/4%)	– 99.5%	3. (1/240%)	– 98%
4. (1/8%)	– 99%	4. (1/480%)	– 60.5%
5. (1/16%)	– 98.5%	5. (1/960%)	– 51%
6. (1/32%)	– 96.5%	6. (1/1920%)	– 8.5%
7. (1/64%)	– 21%	7. (1/3840%)	– 1.5%
8. (1/128%)	– 6%	8. (1/7680%)	– 0
9. (1/256%)	– 3.5%	9. (1/15360%)	– 0

If we compare *A* and *B* in this table it will be seen that while it is true that *B* runs out earlier than *A*, nevertheless the fertilizations in the two series are not proportional to concentrations of sperm; for instance *A* 9 at 1/256 per cent. fertilizes 3.5 per cent. of the eggs, whereas *B* 3 at 1/240 per cent. fertilizes 98 per cent., *B* 4 at 1/480 per cent. fertilized 60.5 per cent., *B* 5 at 1/960 per cent. fertilizes 51 per cent. It is obvious that it is not concentration but *condition* of the sperm that is significant, which comes out with extreme emphasis in a control of this series. In this control, 1 drop of *A* (1 per cent. sperm) was added to 8 drops of the same egg suspension in 1,000 c.c. of sea-water 2 minutes after the other inseminations, thus making a 1/30,000 per cent. (1 × 1/1000 × 1/30) sperm suspension; every egg fertilized; the percentage of cleavage was 100 per cent.

The question then arises, what is this condition of the sperm which causes such loss of fertilizing power? We may note the following points: (1) To bring out the lack of significance of the absolute concentration of the sperm, in several of the experiments with successive half dilutions, counts were made of the numbers of spermatozoa seen in the egg-jelly of members of the series with no fertilizations: Thus on July 16 a series of half dilutions ran out to 0 in the seventh crystal (1/128 per cent. sperm): in ten eggs selected at random from this crystal, an average of 9 spermatozoa was counted in the jelly and in contact with the membrane of these eggs; but, as the upper and lower surfaces

could not be examined, the whole number must have been at least double; in No. 8 of the series, an average of five spermatozoa was counted with each egg; in No. 9 an average of 1.2; No. 10, 1.4; No. 11, 0.9. Similar counts were made in other cases. But in fertilizations under optimum conditions all of the eggs may fertilize in dilutions of sperm so great that it is almost impossible to find spermatozoa in the jelly of the eggs. (2) The spermatozoa are active and the eggs readily fertilizable in such a series as the above. Repeated observations were made on this point; which would be tedious to relate in detail.

It may be noted that in the fertilization under optimum conditions the eggs were first placed in sea-water, and given quantities of sperm then added; whereas in the experiments with successive dilutions eggs were added to sperm suspensions already made up. This suggested that the order of adding eggs and sperm might be of significance in some way. However, this does not appear to be the case.

The possibility remained that the repeated handling of the sperm in successive dilutions decreased their motility. Microscopical examination did not confirm this idea; and subsequent experiments disproved it, as the fundamental factor at least.

Thus it would appear that the only real difference between the optimum and minimum conditions of the fertilizing power of sperm dilutions is a time factor; under what I have called the optimum conditions the final dilution is made from a relatively concentrated sperm suspension in the presence of eggs; but under the conditions of successive dilutions time elaspses before the eggs are added.

Thus in Curve 2 the preparation of the series of sperm dilutions from the original 1 per cent. suspension occupied 22 minutes before the eggs were added. In Curve 1, on the other hand, less than a minute elasped from the time of preparing the 1/100 per cent. and 1/1000 per cent. sperm suspensions used in the last four determinations to the time of their use in inseminating (see p. 234); and the final dilution was made in the presence of the eggs.

The time factor is the real explanation as will be shown immediately. But at first sight it did not seem a very probable

explanation for two reasons: in the first place the time from preparation of the original 1 per cent. sperm suspension to that of addition of eggs is usually less than twenty-five minutes, which is usually considered too short a time for injury to sperm; and in the second place, after the addition of eggs to the sperm-dilution series, in several control experiments the original 1 per cent. sperm suspension was shown to be capable of fertilizing at 1/30,000 of 1 per cent. ($1/2^{15}$ ca.) by addition to eggs in sea-water. *If the sperm suspensions lose their fertilizing power with time, it must be that the significance of time in this respect varies inversely to concentration.* As soon as such a proposition is formulated it is easily tested experimentally, and this was done in a thorough fashion.

4. *Time as a Factor in the Fertilizing Power of Sperm Suspensions of Different Concentrations.*

The experiments under this head were performed in three ways: *A*. A considerable quantity of the sperm suspension to be tested was made up, and divided in several equal parts in a series of bowls; measured equal quantites of the same egg-suspension were then added to members of the series at definite time intervals. This method was followed for sperm dilutions from 1/300 per cent. (between 8 and 9 in the scale) down. *B*. Measured amounts of the more concentrated sperm suspensions were added at time intervals to measured quantities of eggs in equal amounts of sea-water. *C*. Finally, to control the data in section 3, a series of sperm suspensions, made by successive half dilutions as in section 3, was divided in two equal series, and eggs were added at once to the one series, and after a time interval to the second.

A. The following table gives the data under method *A*. The figures at the head of each vertical column give the sperm dilution in fractions of the 1 per cent. sperm suspension; below is given the place of such a sperm suspension in the scale of powers of 2. The figures in the columns give the percentages of fertilizations for inseminations made at the time (age of the suspensions in minutes) indicated at the left. To illustrate the method of experimentation for one column which will serve for all the rest,

TABLE II.

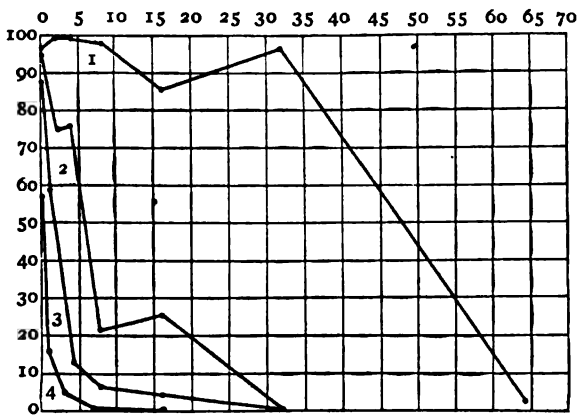
SHOWING RATE OF LOSS OF FERTILIZING POWER OF SPERM SUSPENSIONS.

	1/300	1/600	1/1200	1/2400	1/3000	1/3600	1/6000	1/12000	1/18000	1/24000	1/30000	1/60000	1/120000	1/240000
0	96	100	99.5	95	95.5	97	91	97	....	97.5	88	91.5	58	40
1	....	....	99.5	49.5	.....	52	.....	....	....	.....	....	42	16	6
2	99.5	98.5	.....	....	75	....	80	92.5	98	99.5	59.5	....	.....	....
3	....	....	100	91.5	.....	88.5	.....	.....	....	.....	....	....	4	16
4	99.5	95.5	.....	....	76	.....	34.5	93.66	85.5	49.5	13	15.5	.....	....
6	....	....	93.5	52	.....	80.5	.....	.....	....	.....	....	....	0.5	1
8	98.5	65.5	.....	....	21	....	28.5	54.5	73.5	67	7	17	.....	....
16	85.5	?	99.5	45	26	16.5	.....	.....	14	36	4.5	1	0	0
32	96.5	67.5	30.5	1.5	0	4.5	9	1	0	4	0	0.5	0	0
64	2.5	19	0	0.5	0.5	3.5	2	0	0	4	0	0	0	0
120	8.5	3	.....	....	.....	....	.....	.....	....	.....	....	....	.....	....
	8–9	9–10	10+	11+	11–12	12–	12–13	13–14	14+	14–15	15+	16–	16–17	18–

we shall give the experimental data for 1/30,000 per cent.: August 18, 1914. The eggs of two females were taken at 9.50 A.M. and washed at 10.04, 10.06 and 10.23 (150 c.c. of sea-water being used in each washing). A series of 7 Syracuse crystals was then laid out with 10 c.c. of sea-water in each. To 1 was then added 5 drops of the egg-suspension. A single drop of fresh dry sperm was then added to 333.3 c.c. sea-water at 10.37 making a 1/100 per cent. sperm suspension, and 1 drop of this was added to crystal 1 at 10.37.30 and stirred in by an assistant making a 1/30,000 per cent. (1/100 × 1/10 × 1/30) sperm suspension in presence of eggs. One drop of the 1/100 per cent. sperm was also added to crystals 2–7, which contained no eggs, at 10.38, making 1/30,000 per cent. sperm suspension in each. To No. 2, 5 drops of the same egg-suspension was added at 10.40, to No. 3 at 10.42, to No. 4 at 10.46, to No. 5 at 10.54, to No. 6 at 11.10, to No. 7 at 11.42. At 2.30 P.M. my assistant, Mr. Cohn, then estimated the percentages of segmented eggs in each crystal, by first thoroughly mixing the eggs, then assembling them, taking a sample, and making two counts of 100 each, which were averaged.

The table shows (1) that the effect of time up to 64 minutes is to diminish the fertilizing power of the suspensions at every dilution represented. (2) That the rate of loss of fertilizing

power increases with dilution, *i. e.*, the effect of time varies inversely to concentration of sperm. This is brought out very clearly by the following curves (Fig. 3) of loss of fertilizing



CURVE 3.

power of sperm suspensions at different concentrations. The abscissæ represent age of sperm suspensions in minutes; the ordinates represent fertilizing power as expressed in percentages of segmenting eggs. Each curve stands for a given sperm dilution. Curve 1 represents loss of fertilizing power of a 1/300 per cent. sperm suspension, curve 2 of a 1/3000 per cent., curve 3 of a 1/30,000 per cent. and curve 4 of a 1/120,000 per cent. sperm suspension.

B. On August 6, I prepared a series of seven sperm dilutions in powers of 4 from 1 per cent. to 1/4096 per cent. Each of these was then used to fertilize a measured quantity of egg-suspension at the intervals given in Table III.

For the fertilizations 10 c.c. sea-water was measured out in advance in Syracuse crystals and 5 drops of a 5 per cent. egg suspension added to each. For each fertilization 1 drop of sperm was added and stirred in. It will be observed that 1 per cent. sperm lost none of its fertilizing power so far as this test went; 1/4 per cent. fell off from 96.5 per cent. to 16.3 per cent.; 1/16 per cent. from 46.5 per cent. to 0; 1/64 per cent. from 0.5 per cent. to 0 in the second place; whereas the greatest dilutions did

TABLE III.

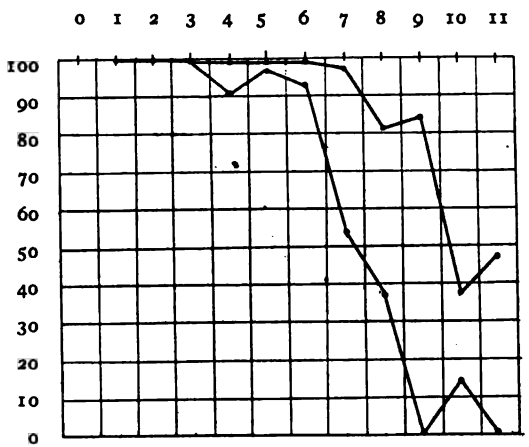
Sperm Dilution.	Made at	Fertilizations.				
		A.	*B.*	*C.*	*D.*	*E.*
		2.50 P.M.	3.01 P.M.	3.16 P.M.	3.35 P.M.	4.03 P.M.
1. 1%.....	2.20 P.M.	99 %	100%	100 %	99.5%	99.5%
2. 1/4%.....	2.28 P.M.	96.5%	68%	61.5%	18.5%	16.3%
3. 1/16%....	2.30 P.M.	46.5%	18%	8.5%	4 %	
4. 1/64%....	2.32 P.M.	0.5%	0%	0%	0 %	
5. 1/256%...	2.34 P.M.	0	0%	0%		
6. 1/1024%..	2.36 P.M.	0	0%			
7. 1/4096%..	2.39 P.M.					

not fertilize at all. It should of course be noted that the sperm suspensions used were diluted 300 times in the actual insemination (10 c.c. = 300 drops of sea-water, and one drop sperm added).

Thus time is an important factor in the fertilizing power of sperm dilutions from 1/4 per cent. down. The matter cannot be stated with great accuracy, but we can say in general (referring to Table II.) that sperm suspensions from 1/300 per cent. to 1/1200 per cent. lose their fertilizing power nearly completely in 64 minutes; from 1/2400 to 1/24,000 in 32 minutes; from 1/30,000 to 1/60,000 in 16 minutes; from 1/120,000 to 1/240,000 in 6 minutes. Table III. shows loss of fertilizing power of higher concentrations by a different method.

C. We are now in a position to understand the principal reason why the curves of successive half or quarter dilutions of a 1 per cent. sperm suspension run off so rapidly. The reason is that the preparation of the series requires time, 10 to 20 or more minutes. In the early experiments of this kind the significance of brief periods of time was not recognized, and so no time records were kept; but I have 13 curves with accurate time records. Of these I reproduce only two (Fig. 4). For this experiment (Sept. 5), (1) 6.6 c.c. of 1 per cent. sperm was prepared, 9.30 A.M. (2) 4 c.c. of 1 was transferred to a Syracuse watch crystal and 4 c.c. of sea-water added (= ½ per cent.). (3) 4 c.c. of 2 was transferred to crystal 3 and 4 c.c. sea-water added (= ¼ per cent.) and this was continued to 12 numbers (finished at 9.39.30 A.M.). The suspensions 2–12 was then divided in two equal amounts of 2 c.c. each, making series *A* and *B*. To each crystal of series *A* 2 drops of a 1 per cent. egg-suspension were

added at 9.47, that is, 17 minutes after the 1 per cent. sperm was prepared. Twenty-eight minutes later (10.15 A.M.) two drops of the same egg suspension were added to series *B*. The only difference between series *A* and *B* is the time factor. The percentages of cleavages were counted for both series, and the plotted results given the curves. Considerable loss of fertilizing power



CURVE 4.

has occurred in series *B* as compared with series *A*. Now, if we compare these time intervals with those given in Table II. we see that, in the curve of series *B* the last number, which is a $1/2^{11}$ or 1/2048 per cent. sperm suspension, loses its fertilizing power completely in 36 minutes (*i. e.*, from 9.39.30 to 10.15.30) which corresponds very well with the rate of loss of fertilizing power in a 1/2400 per cent. sperm suspension. This agreement is rather closer than usual; in some cases the series of ½ dilutions ran out at higher concentrations in about the same time; but in no case, I think, did they require more time. This suggests some possible stimulating effect of the successive changes which causes the spermatozoa to lose their fertilizing power more rapidly than under the time factor alone.

Gemmill (1900) observed that the duration of vitality of spermatozoa of sea-urchins and limpets tested by their movements or by the fertilizing capacity, varies greatly "according to the amount of sperm used in proportion to the volume of sea-

water in which it was shed." "When a small quantity of sperm was mixed with a large quantity of sea-water, the duration of vitality of the spermatozoa is short, but when the converse proportions are used, it is greatly lengthened." "By taking sperm from a sea-urchin and mixing it in different vessels with different quantities of sea-water, one obtains sets of spermatozoa, which will retain their vitality for a rising series of terms, *e. g.*, 8, 12, 16, 24, 48 and 72 hours. For the longest term, the proportion of spermatic fluid to sea-water should be not less than 1 to 10."

Gemmill was thus dealing with the same phenomenon with which we are concerned. He gives, however, no exact quantitative data and relatively few experiments were performed. He attributes the results to (1) greater activity of the spermatozoa, and consequent earlier exhaustion in the more dilute suspensions and (2) to dilution of the "spermatic fluid" by which he supposes the spermatozoa to be nourished.

5. *Other Factors in the Fertilizing Power of Sperm Suspensions.*

In the large number of experiments carried out to test the fertilizing power of sperm suspensions the general form of the curves is remarkably constant. Some, however, are quite irregular, and it was never possible to get *exactly* the same curve in the repetition of any experiment. A few of the irregularities may conceivably be due to error, as for instance the accidental presence of some toxic substance in one of the dishes of a series, though painstaking care was used to avoid such sources of error. The failure to obtain exactly the same curve in different experiments is no doubt also due in part to the natural variability of different lots of eggs and sperm.

In an attempt to discover the sources of variation and error, the effect of egg concentration, *i. e.*, the absolute quantity of eggs in a given bulk of a sperm suspension of given strength was tested. On the whole the effect of egg-concentration was found to be relatively small within so wide a range that it cannot be regarded as a large factor in the variability of the curves; because the egg-concentration of the curves was always below the point where it was demonstrably a limiting factor. Tests were made of sperm suspensions ranging from 1/62.5 per cent. to 1/8,000

per cent. But it was only from about 1/500 per cent. down that any considerable effect was observed within the range of egg concentration employed.

The method of the experiments tabulated (Table IV.) may be given for 1/500 per cent. sperm as it was the same for the others August 31: A quantity of 1/500 per cent. sperm suspension was freshly prepared 10.54.30 A.M., 2 c.c. of this was then placed in each of seven crystals (1–7). From 10.56.30 to 10.59 A.M. eggs were added as follows: to 1, one drop of a 1.75 per cent. egg-suspension, to 2 two drops, to 3 four drops, to 4 eight drops, to 5 sixteen drops, to 6 one c.c., to 7 two c.c. The numbers in the table give the percentages of segmented eggs. The tests with 1/1,000 per cent. and 1/2,000 per cent. sperm were made with the same egg suspension. For the tests with 1/4,000 per cent. and 1/8,000 per cent. sperm a 3.3 per cent. egg-suspension was used. Thus for each series the egg-concentration is approximately doubled in successive numbers of the series (in No. 7 = 64 times No. 1).

TABLE IV.

EFFECT OF EGG-CONCENTRATION ON THE FERTILIZING POWER OF SPERM SUSPENSIONS.

Sperm Suspensions.	Egg-suspensions.						
	1.	2.	3.	4.	5.	6.	7.
1/500%	100	99	99.5	97	93.5	82.5	56
1/1000%	97.5	94.5	93	76.5	48?	76.5	33.5
1/2000%	96.5	83.5	75	72.5	42.5	36	32
1/4000%	79.5	66.5	42.5	47.5	12.5	16	4.5
1/8000%	46.5	52	80?	30.66	27	15.5	7.5

The percentages of fertilization fall off in each of these sperm suspensions with increase of egg-concentration, and the amount of falling off increases in general with the dilution of the sperm. There was certainly no numerical deficiency of spermatozoa in the highest egg-concentrations; the reason for the falling off therefore appears rather obscure, and as it is not involved in the present problem, I shall not discuss it here. But as the egg-concentration employed in any of the preceding experiments did not exceed that of column 3, and the same egg-concentration

was always employed throughout any experiment, it is obvious that the effect to be attributed to the egg-concentration employed in the preceding experiments is very small.

III. Discussion.

Within a wide limit of egg-concentration the important factors in fertilizing power of sperm suspensions are: (1) concentration, (2) time. A third factor, which is not of equal significance to the other two, is the given variability of the reproductive elements. Such variability attaches of course both to ova and spermatozoa; in general it will affect only absolute values for given combinations, and not at all the relative values found in any single experiment. Moreover, as it is a chance factor, it will tend to be eliminated in a series of determinations. Fortunately both eggs and spermatozoa of *Arbacia* are relatively very constant materials if care be taken to wash the eggs thoroughly, and if the factors of concentration and time are fairly constant for the sperm. For the eggs these two latter factors are of such slight importance within the given limits as to be practically negligible. The significance of the concentration factor for the fertilizing power of sperm is of course obvious without discussion. We therefore turn to the time factor.

The most significant aspects of the time factor are, first, the unexpectedly rapid rate of loss of fertilizing power of sperm suspensions, and second the increase of rate of loss with dilution. There are but two ways of explaining these facts: either (1) the motility of the spermatozoa is quickly reduced in sperm suspensions to such an extent that they cannot bore into the egg or (2) the spermatozoa lose some substance essential for the fertilization reaction.

The following are the objections to the first alternative: (*a*) Microscopical examination lends it no support; I have repeatedly observed, that fertilizing power of sperm cannot be expressed either in terms of motility, or of success in penetrating the jelly of the egg and coming in contact with the membrane. In the experiments on successive half dilutions (p. 238) I kept records, in several series, of the numbers of spermatozoa in the jelly of unfertilized eggs, and found in some cases an average of 9

spermatozoa visible in the jelly, or on the membrane of certain lots of eggs none of which had fertilized; this could not be more than half of the spermatozoa in association with such eggs; and other observations made immediately after insemination demonstrated the high degree of motility of spermatozoa of entirely barren sperm suspensions.[1]

These observations contrast in the most striking manner with the fact that not a single spermatozoön can be seen in the jelly of eggs fertilized with highly dilute fresh sperm suspensions, where, nevertheless, nearly every egg may be fertilized.

(*b*) Penetration of the egg is not solely a function of motility of the spermatozoön. Penetration follows, as a matter of fact, after the fertilization reaction has begun, and it is due to the inception of such reaction, not the reverse as is commonly assumed.[2] In *Nereis*, as I have previously described, penetration does not take place until 45 to 50 minutes after insemination and the initiation of the fertilization reaction. The facts described in this paper show that in *Arbacia* no penetration takes place unless the sperm has started the fertilization reaction; if this does not take place, the spermatozoön remains external, however active it may be. And if it does occur the initiating spermatozoön is speedily engulfed by the egg.

(*c*) It is not easily understood on this theory why dilute sperm suspensions should lose their fertilizing power more rapidly than

[1] Glaser's experiments (1915) would bear the interpretation that, in those cases of normal insemination described by him in which fertilization does not occur except in the presence of several spermatozoa for each egg, the time factor which I have just described was operative. In other words that the majority of the spermatozoa in question had lost their receptors. But in the absence of exact data as to age and concentration of the sperm suspension, it cannot be asserted that this is the correct interpretation although I obtain exactly the same results in my time series (p. 238). My dilution experiments prove beyond a doubt that a single spermatozoon suffices for the whole process of fertilization under optimum conditions (defined on p. 233). Glaser's experiments, however, raise the question whether the efficacy of heavy insemination in the case of a stale sperm suspension is due to mass action, or to the survival of a small percentage of effective spermatozoa? So far as I can see this question can not be answered on the basis of our present information.

[2] Spermatozoa may penetrate into unripe ovocytes in some cases, as has been noted by several observers; in such a case there is no fertilization reaction. In the present experiments the unfertilized eggs were not penetrated by the spermatozoa.

more concentrated suspensions;[1] the relative freedom from CO_2 and other sperm excreta should favor a longer continuation of their motility in the dilute suspensions rather than the reverse.

(*d*) Moreover, in general the results of recent fertilization studies such as the antagonistic action of sperm suspensions of different phyla, inhibition of fertilization in the presence of blood of the species, or in the absence of certain ions (Loeb, '14), or again the sterility in certain self-fertilizations, and finally the inability of spermatozoa to penetrate fertilized eggs, unite in demonstrating the relative lack of significance of motility as such.

We come therefore to the conclusion that the individual spermatozoa in suspension tend to lose their fertilizing material, so that an increasing proportion of these spermatozoa become absolutely ineffective whatever their motility. This conclusion is in agreement with all the data of the foregoing experiments, and seems to be the only one competent to explain the results.

The following questions arise: (1) Whether the loss of this substance by the sperm is a mere process of diffusion or an active secretion? (2) Can the substance be recovered from the fluid of the suspension, or can its presence in the fluid be demonstrated in any way?

As regards the first question: In the case of the ova we know that the external jelly-covering is loaded with sperm-agglutinating substance which diffuses into the sea-water continuously. It is theoretically possible, at least, to apply a similar conception to the spermatozoön, although no such covering is demonstrable. The more rapid loss of fertilizing power in the greater dilutions would be consistent with this interpretation. From this point of view we would have to regard the sperm head as covered superficially with a layer of fertilizing material, like the phosphorus on a match. Such a conception is by no means impossible. On the other hand the fact that dilutions reached by a series of successive half-dilutions from 1 per cent. lose their

[1] Gemmill (1900) observed the same phenomenon and concluded that the more rapid exhaustion of spermatozoa in dilute suspensions is due to dilution of a hypothetical nutritive medium which keeps the spermatozoa of concentrated suspensions in a vigorous condition. This explanation comes back to the principle of loss of motility, so far as it relates to fertilizing power.

fertilizing power more rapidly than the same dilutions made in one stroke, indicates that successive stimulation hastens the loss, which therefore appears more in the nature of a secretion or a discharge than mere diffusion. The source of the substance must ultimately be the sperm cell itself, and it is quite possible that, as in the case of the egg, there is both a superficial layer and an internal supply.

It must be admitted that the data are inadequate to answer this problem. The statement of the problem can therefore serve only to bring out the resemblance between the spermatozoön and the ovum in respect to the existence of a fertilizing substance in each, the fertilizin in the case of the ovum and the sperm receptors in the case of the spermatozoön, and also the possible resemblance in respect to the disposition of the substances in each. It certainly is an interesting parallelism that both cells contain a substance necessary to fertilization, which may be lost in the sea-water.

The most interesting and crucial question of course concerns the possibility of detecting this lost substance in the fluids of the suspensions. If such a substance actually occurs in the fluid it should have the property of fertilizing ova; unless it can be detected by this property, we have no other indicator for it. So far I have not been able to make even a beginning on this problem. As is well known a number of experimenters have attempted without success to derive a fertilizing medium from spermatozoa. It has been suggested by Loeb that the reason for the failure to secure an extract of spermatozoa that will fertilize is that the motile power of the spermatozoön is needed to carry the effective substance into the egg. But it may equally well be that the methods hitherto employed have been too brutal; the substance may well be too labile to withstand extraction by ether, etc.

My results strongly suggest, if they do not prove, that such a substance must be present in the fluid of sperm suspensions of *Arbacia*, and they therefore suggest other methods for securing it for testing. We must bear in mind that it can form only an extremely small proportion of the entire spermatozoön, as proved by morphological considerations alone, and that it must

be superficial in position and easily detached as proved by its effectiveness before the spermatozoön penetrates. Extracts of the entire spermatozoön must contain numerous other substances which may neutralize its effectiveness.

The difficulty of the investigation as shown by my experiments is that it is liberated only very slowly in concentrated suspensions and that its amount in dilute suspensions would presumably be too slight to be effective. Some means can probably be devised for liberating it in concentrated sperm suspensions and freeing it of the spermatozoa for testing.

Finally I may point out that the conclusion that spermatozoa lose a substance necessary for the exercise of their fertilizing power is consistent with my own point of view of the mechanism of fertilization as well as with Loeb's. From my point of view the spermatozoön loses its receptors, viz., the substance that activates the fertilizin of the egg; from Loeb's point of view the spermatozoön loses its lysin, the substance that corrodes (cytolyzes) the egg.

My previous experiments had shown that eggs produce a certain substance in sea-water (fertilizin) which is necessary for their fertilization; fertilized eggs no longer produce this substance and are incapable of fertilization. Both eggs and spermatozoa therefore contain substances, more or less liable to loss, which are necessary for fertilization. The mechanism of fertilization cannot possibly, therefore, be regarded in the simple manner postulated by Loeb's theory. The existence of parthenogenesis demonstrates the efficacy under given conditions of the egg-substance alone; we must therefore regard the spermatic substance essentially as an activator of the fertilizin of the egg.

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THE PRODUCTION OF SPERM ISO-AGGLUTININS BY OVA

I. If one allows unfertilized eggs of *Arbacia* to stand in a quantity of sea-water that does not exceed about ten times the volume of the eggs, the sea-water soon becomes perceptibly tinged with the red coloring matter of the eggs. If now a few drops of such supernatant sea-water be added to 2 or 3 c.c. of a milky suspension of active sperm of the same species, a strong agglutination of the sperms immediately ensues, producing sperm-masses easily visible to the naked eye. In the course of three to five minutes reversal takes place, the masses become converted into their constituent cells, and considerable activity may be observed after the reversal on microscopical examination. The substance which produces this phenomenon may be called a sperm agglutinin, and since it is produced by the same species, an iso-agglutinin.

This basic phenomenon was studied in three ways: (1) In a test tube or vial containing 2 or 3 c.c. of a milky sperm suspension, as above described. (2) Drops of the sperm suspension and of the egg-extract may be placed side by side on a slide and connected under the microscope, so as to observe the inception and progress of the phenomenon. (3) The best method of studying the detail of the phenomenon is to mount some of the sperm suspension beneath a long cover glass supported by glass rods 0.5 to 1 mm. in diameter, and to inject a drop of the egg-extract with a capillary pipette into the suspension. If this be observed under a low power of the microscope, one sees that the drop fills with sperm exhibiting increase of activity, which immediately agglutinate into small masses, which then unite with great rapidity to form larger masses spaced throughout the drop. At the margin of the drop and the sperm suspension a ring of agglutinated sperms forms which ruptures in numerous places, each segment contracting to form a mass, so that the continuou becomes a chain of beads visible to the eye. The ring forms in a second or tw breaks into masses in two or three se The agglutination is totally reversible, as the visible effect is concerned, and the spermatozoa, or some of them, appear gain full activity; moreover, the sperm between the masses are in active mov throughout.

The reversal of the reaction consists resolution of the agglutinated masse their individual cells, but this does not a return to their original physiological tion; for if the agglutinating solution extract) be sufficiently strong, after a of intense stimulation followed by aggl tion and reversal, the movements of the matozoa gradually cease entirely and minutes they are no longer capable of izing ova dropped in with them.

The agglutination is a factor of (1 activity and relative density of the spe zoa in the suspension, and (2) of the st of the agglutinating solution. The active the sperms and the greater the d of the suspension (within certain limit more rapid is the onset of the aggluti and the larger the agglutinated masses. agglutinating medium may of course be to vary in strength, and hence in aggl ting power by various means, *e. g.*, by cr ovaries and eggs in about their own b sea-water, or extracting in distilled wate powerful solutions may be made.

The agglutinating agent is not readi stroyed by heat. After boiling and mai ing at a temperature of 95° to 98° C. for 70 minutes, the agglutinating action strong extract was very much reduce was not entirely lost.

No other tissue of the sea-urchin, so

rved, produces an agglutinating agent for perm of the species: (1) The serum from body-cavity, whether of males or females, tirely neutral and the spermatozoa swim normally. But the serum forms a power- gent for extracting the agglutinin from eggs after the latter are removed from ovary, though in the intact animal in h the eggs are separated from the serum he ovarian membrane no extraction of tinin takes place. (2) Large pieces of ntestine were cut up in sea-water or in lled water, the latter especially extracting ed matters in large quantity; but no n agglutinin could be detected in the te.

e agglutination reaction fixes the agglu-, as is readily proved by the fact that it pears from an agglutinated sperm sus- ion, if not present in excess. Whereas an l dilution with sea-water of the original xtract is still highly agglutinative. The tinative substance, therefore, presumably s into combination with some sperm sub- e acting in this respect similarly to its gen.

The results are essentially the same for *is*, except that the visible agglutination t reversible to the same extent, a differ- which is probably of purely secondary ficance. Moreover, the agglutinin is pro- d by the eggs in sea-water only in small tities before fertilization, but in very quantities at the moment of fertiliza-

The eggs of *Nereis* when obtained are e stage of the germinal vesicle, whereas of *Arbacia* have formed both polar es, and this is probably correlated with different rates of secretion of the agglu-. The agglutinin of *Nereis* is moreover more thermolabile, being destroyed at 0. in ten minutes. In *Nereis* also it is ult to test other tissues satisfactorily, as nimal is practically a bag of eggs when ally mature, but such tests as were made cate that the eggs alone secrete the agglu-. In other respects *Nereis* is a more rable form for study, as the sensitiveness e spermatozoa is unsurpassed.

I. As regards the important question of specificity, it seems very probable *a priori* that a substance which is produced only by the eggs of a given species, and which is agglutinative for the sperm of that species should be specific. Now the egg-extract of *Nereis*, which agglutinates *Nereis* sperm, is entirely devoid of agglutinating effect on *Arbacia* sperm, so that there appears to be specificity of the *Nereis* agglutinin to this extent at least. But the egg-extract of *Arbacia*, on the other hand, is extremely toxic and agglutinative for *Nereis* sperm, so that in this case either the agglutinin from *Arbacia* eggs is not specific for its own sperm, or the egg-extract contains, in addition to the specific agglutinin, another toxic substance. The latter alternative is probable, as is shown by the fact that the serum of the cœlomic fluid of *Arbacia*, which is not in the least toxic or agglutinative for *Arbacia* sperm, is as strong an agglutinative agent for *Nereis* sperm as the egg-extract of *Arbacia*. The presence of at least two sperm agglutinating substances in *Arbacia* is therefore certain, viz., one in the egg-extract, which is agglutinative for *Arbacia* sperm, and one in the cœlomic fluid not agglutinative for *Arbacia* sperm but agglutinative for *Nereis* sperm. I assume, therefore, provisionally, that both are present in the egg-extract, and that the sperm iso-agglutinin of *Arbacia* eggs is probably specific.

This assumption is rendered more probable by an observation made only once, and at the end of the season, so that its repetition this year is impossible. I had preserved a strong egg-extract of *Arbacia* for seventeen days. This had been tested at various times with *Arbacia* sperm and found to retain its agglutinating power without any observable diminution. But on the seventeenth day, when the last *Nereis* of the season was brought in, I was surprised to find that the egg-extract in question had lost its agglutinating power on the *Nereis* sperm. The same sperm was agglutinable with an egg-extract of *Nereis* eight days old; so that the difficulty could not be with the sperm. And the same egg-extract of *Arbacia*, on retesting, was found to retain its agglutinating power on the *Arbacia* sperm appar-

ently unaltered. The specific agglutinin is therefore relatively stable with reference to the conditions involved, and the non-specific agglutinin or toxin is relatively labile. It might, therefore, be possible to separate them also by heat or other means.

One of these would be to fix the non-specific agglutinin of *Arbacia* egg-extract with *Nereis* sperm, and test the filtrate for the persistence of the assumed specific agglutinin with *Arbacia* sperm. If the *Arbacia* agglutinin were found to persist after the filtrate had lost its *Nereis* agglutinating power, the presence of the two agents in the egg-extract would be demonstrated. Or if, after completely fixing the specific agglutinin of *Arbacia* egg-extract with *Arbacia* sperm, a *Nereis* agglutinating agent were still found, the same conclusion would be inevitable. Unfortunately, by the time this stage of the analysis was reached, the season for both forms was over, and this experiment must be postponed for a year.

IV. The egg-extracts contain not only an agglutinin for the spermatozoa, but also an aggregative agent, *i. e.*, a substance towards which the spermatozoa are positively chemotactic. This may be readily demonstrated by the form of the reaction when a drop of the fluid to be tested is injected into a sperm suspension beneath a raised cover glass. If an aggregative agent be present, a ring of spermatozoa forms at or within the margin of the drop, depending on the strength of the agent, and a clear zone arises between this ring and the general sperm suspension. The clear zone is produced by migration of spermatozoa to the ring; in case the agent is very strong the ring expands, owing to immigration of spermatozoa, but the clear zone is never obliterated, no matter how much the ring may expand. In the case of *Nereis*, which has unusually large spermatozoa, the passage of spermatozoa across the clear zone to the ring may be readily studied under a low power of the microscope, and it gives the impression of a regular rain falling on the ring.

In the case of *Nereis* all acids tested are aggregating agents (CO_2, H_2SO_4, HNO_3, HCl and $CH_3 \cdot COOH$ were studied), but do not agglutinate, and alkalis (K, OH and NaOH only studied) are agglutinative but n gregative. The sperm of *Nereis* tremely sensitive to acids, reacting pos to $N/1{,}000$ H_2SO_4, HCl, HNO_3, and N acetic, and to 1/200 dilution of a sat solution of CO_2 in sea-water. The phen of aggregation were therefore studied ticularly in the case of *Nereis*. The tiveness of *Arbacia* sperm to acids is t four times less than that of *Nereis* sper the reactions are in the same sense.

If an acid or other aggregative agen be present in the drop tested in the s sion beneath a raised cover slip, th which forms is perfectly continuous a individual spermatozoa are separate. agglutinin as well as an aggregative ag present, the ring forms and breaks u agglutinated masses. If the agglutinin be present no ring proper forms, or t no outer clear zone, and agglutinated form within the drop as described. Th secretions give the double reaction.

V. Von Dungern's experiments (*Zei allgem. Physiologie*, I., pp. 34–55, 190 the only ones, so far as I know, in wh production of sperm agglutinins by o investigated, and he discovered only agglutinins, no iso-agglutinins. He d deed, describe the loss of motility of s tozoa in egg-extracts of the same speci he entirely missed the phenomenon of tination and its reversal. He reveals t son for this failure by his remark t always examined for the effect of the poison" about half an hour after its a to the sperm; but the phenomenon of tination and its reversal are comple about five minutes.

Von Dungern also made experiments production of immune sera by inject ova and spermatozoa separately into and found that both caused the producti sperm agglutinin in the rabbit's serum. this he concludes that both kinds of re tion elements possess chemically id complexes of molecules in the prot While this may be admitted as at least probable conclusion, his farther con that fertilization does not depend up

etween ovum and sperm,
y the similarity of their
vell founded, for the egg
ed chemical system, and
molecules antagonistic to
on Dungern's experiments
ains some that are not.
s of sperm iso-agglutinins
sibility of an explanation
fertilization on the basis
ning antigens and anti-
lutinins are specific, as is so strongly suggested by the experiments. The union of ovum and spermatozoon is not a process in which the sperm penetrates by virtue of its mechanical properties, but one in which a peculiarly intimate and specific biochemical reaction plays the chief rôle. A later publication will give the details of the experiments and a more complete analysis of the behavior of the spermatozoa in fertilization.

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BREEDING HABITS OF THE HETERONEREIS FORM OF NEREIS LIMBATA AT WOODS HOLE, MASS.

FRANK R. LILLIE AND E. E. JUST.

The senior author has used the eggs of the pelagic form of *Nereis limbata* in studies on fertilization for several years. During the seasons of 1911 and 1912 collections were made each night by the junior author, and records were kept which reveal considerable uniformity in swarming periods in accordance with the phases of the moon. In addition advantage was taken of the opportunity to study certain other features of breeding behavior. These observations are brought together in a paper partly for the sake of such general interest as they may possess and partly for the information of those who may have occasion to use this form for investigations.

The animals may be taken after sunset on certain nights, in general during the "dark of the moon," in the months of June, July, August and September. They appear swimming near the surface of the water very soon after sunset, and may be attracted by the light of a lantern and readily caught with a small hand net. The swarming usually begins with the appearance of a few males, readily distinguished by their bright red anterior segments and white sexual segments, darting rapidly through the water in curved paths in and out of the circle of light cast by the lantern. The much larger females then begin to appear, usually in smaller numbers, swimming laboriously through the water. Both sexes rapidly increase in numbers during the next fifteen minutes, and in the case of a large swarm there may be hundreds of males in sight at one time, though the number of females to be seen at one time rarely exceeds ten or a dozen.[1] In about 45 minutes the numbers begin to decrease and

[1] At the height of some runs, as for instance, on the evenings of June 12, July 5 to 8, and August 6–10, 1912 (see curves 6, 7 and 1), great numbers of females appeared. On one occasion at least a liter of females was secured in ten minutes, several being caught with each sweep of the net. Within the circle of the light on such nights as many as fifty females may be seen at once.

in an hour, or an hour and a half, all have disappeared for the night. Next night the scene may be repeated. The females are a fresh crop each night, as will be seen from the description below, though the same males may presumably appear on several successive nights, a circumstance which, if true, will partly explain the usual great numerical preponderance of males.[2]

In any given night the males invariably appear first. As the females appear in increasing numbers, the males gradually grow fewer. During the first nights of a run, as well as towards the end, the males are relatively more abundant. On nights of the greatest swarms, when the females appear in great numbers, the disproportion is perhaps not as noticeable. It seems, however, that there is no definite place in the run at which this sex ratio changes, although there are some nights throughout the summer when more females than males are caught.

During the light of the moon, with exceptions noted beyond, no *Nereis* are to be found. They therefore occur in four periods or "runs," during the summer, corresponding to the lunar cycles in the months of June, July, August and September. Each run begins near the time of the full moon, increases to a maximum during successive nights and sinks to a low point about the time of the third quarter, then again rises and falls to extinction shortly after the new moon. Each run is therefore typically divided in two sub-runs, or the curve of nightly numbers during a run is bimodal, with a deep depression about the time of the third quarter.

When a female appears she is soon surrounded by several males, if the latter are abundant, which swim rapidly in narrow circles about her. In a little while they begin to shed sperm, rendering the water milky, and soon she begins to shed her eggs, shrinking in bulk as she does so, until, a mere shadow of her former self, she sinks slowly in the water to die. We have never succeeded in keeping females alive for more than a few days after the eggs are shed.

[2] The worms are eaten voraciously by certain fish, as we ascertained in 1911 when *Nereis* were kept in aquaria with *Fundulus*. During August, 1911, it was often noted that fish attracted by the light seemed to keep down the number of *Nereis* caught. The slowly moving females were always more easily caught by the fish. This may therefore be another cause that influences the sex ratio.

If the animals are to be kept over night for work the next day, they should be placed in separate finger bowls, preferably one for each individual, but in any case never mixing the sexes. If then the bowls are placed in a cool place, as on a water table with water flowing around them, a considerable proportion of the females and all of the males will retain their sexual products over night, and the eggs may be fertilized as desired. One great advantage of *Nereis* for experimental work is that each swarming individual is always fully mature, and contains no immature sexual elements whatever.

I. The Causes of the Sperm-shedding and Egg-shedding Reflexes.

An incidental observation of the senior author put us on the track of the immediate cause of the sperm-shedding reflex. It should be noted that males retain the sperm very tenaciously as a rule, even after days in the laboratory. One day a male was dropped accidentally in a bowl of sea water which had previously contained a female. He immediately began to shed sperm, and swam round and round in the bowl very rapidly casting sperm until the entire 200 c.c. was opalescent. This suggested that the effect of the presence of the female upon the male was exerted not through sight or touch, but through some chemical stimulus, and a few simple experiments demonstrated this conclusively.

If a ripe female be kept in a finger bowl containing about 125 c.c. of sea-water for several hours, the water will usually be found to be charged with a substance which immediately incites the sperm-shedding reflex of the male. Thus on June 17, 1911, the following test was made with about 125 c.c. of sea-water in which a female had been kept over night. A small quantity of this charged sea-water was placed in a Syracuse watch crystal, and a male was dropped in (8:15 A.M.); at once he shed sperm. He was then returned to fresh sea-water and ceased shedding. At 10:30 he was again dropped into a watch crystal of the charged sea-water and again shed sperm; in a few seconds he was transferred to fresh sea-water, and he ceased shedding sperm. Again at 2:07 he was transferred to another supply of the charged sea-water, and again shed sperm. Returned to fresh sea-water

he ceased. At 2:20 he was tested again with positive results for the fourth time, and a little later was tested once more with water charged by another female and shed sperm once more.[1]

Thus each time that this animal was placed in sea-water that had contained a female for a certain length of time he responded instantaneously, and each time that he was removed and put in fresh sea-water he ceased to shed sperm.

This reaction is almost mechanical in its regularity, and can be secured with almost any male in good condition. Cases were found in which certain females apparently did not give off the effective substance, but they were very rare. As a general thing a large fully ripe female will so charge 125 c.c. of sea-water in an hour's time that any active mature male will give the sperm-shedding reflex almost instantaneously on being dropped in after the removal of the female, whereas even repeated transfers from one bowl of fresh sea-water to another causes no such response. If a male and female be placed together in a bowl of fresh sea-water they appear to stimulate one another very quickly, but it is usually several minutes, at least in the case of animals that have been kept in the laboratory over night, before the male begins to shed sperm; and the female never sheds her eggs until after the male has begun to shed sperm.

We have seen that some kind of emanation from the female incites the male to shed sperm. In the case of the female, however, it is not any comparable emanation from the male, but the presence of sperm in the sea-water that incites the shedding of eggs. The females are much more apt to shed their eggs spontaneously than the males to shed sperm. Apparently, when fully mature, many females are unable to hold the eggs back. But, in the case of those that do, it is impossible to cause the egg-shedding reflex by putting them in water that has contained males; on the other hand, the addition of a certain quantity of

[1] Mechanical shock, killing fluids, alcohol and fresh water, may cause the worms to shed their sexual products; probably any sufficiently strong shock may act similarly. But by slow addition of alcohol to the sea-water the animals may be stupefied and die extended without shedding the sexual products. Worms freshly caught react more surely to secretions of the female than those that have been in the laboratory over 18 hours. The reactions are not so definite in the late afternoon of the day after capture, and this is associated with a general lowering of tone.

sperm to the water containing the female will usually cause shedding of the eggs.

There is thus a very precise form of sexual behavior which was tested and demonstrated repeatedly during the seasons of 1911 and 1912. No attempt has been made to study the subject at all exhaustively as our attention was directed to other problems, but a few experiments were made dealing with the male reflex that throw some more light on the matter.

In the first place it is probable that the emanation of the female that incites the male to shed sperm comes mainly from the eggs. This could be shown in two ways: (1) Spent females, *i. e.*, those that have shed their eggs, do not produce the inciting substance in quantities comparable to the production of egg-bearing females. It was possible to obtain some effects by keeping four or five spent females in a small quantity of sea-water over night; but the effect on the male was so slight as probably to be attributable to the few remaining eggs in the female, or to egg secretions persisting in their bodies. Moreover mature males would not respond to the presence of immature atokous females. (2) The eggs alone produce the inciting substance quite rapidly; thus in one experiment about one third of the eggs of a single female charged about 10 c.c. of sea-water in 80 minutes so that a male shed sperm quite freely on being put in some of this water. Transferring to successive bowls of sea-water caused no reaction. The same male shed yet more freely on being transferred to the same quantity of sea-water from a bowl of 125 c.c. in which a mature female had stood over night. This was repeated with several variations always with positive results.

The tests showed that the eggs alone were considerably more effective than the spent females. The production of the stimulating substance should therefore be attributed mainly to the eggs.

The problem of the nature of the emanation that stimulates the males has received inadequate attention; but some experiments were made which narrow the field down considerably. In the first place the reaction is not due to the CO_2 excretion of the females. This follows from the fact that males do not produce any stimulating substance, though they excrete CO_2. A series

of tests was also made with various dilutions of sea-water supersaturated with CO_2, all to no effect.[1] In the next place it is not due to any substance common to the females of marine animals. Tests were made with sea-water charged in one case with the emanations of *Fundulus*, in another case with the emanations of another polychæte, *Chætopterus*. Males placed in portions of such charged sea-water gave no reaction whatever, but the same males placed in a portion of sea-water that had contained a female *Nereis* over night immediately shed sperm abundantly. The mature males of *Nereis limbata* will not react even to the ripe pelagic females of a nearly related form (*N. megalops*).

It is therefore probable that the substance in question is specific. In this there is involved no new biological principle. It is well known that the males of certain butterflies, for instance, are attracted even from long distances to the females by virtue of some air-borne emanation that is certainly specific. And the same principle must also be involved in the relations of males and females in many other instances.

Another property of the exciting substance is its lability. This is shown by its disappearance on standing in the course of about three days, and by its destruction by heat. As regards the first point, the following experiment may be noted: June 24, 1911. Four samples of sea-water charged by *Nereis* females were tested, each of which had been effective when fresh: *a* was 7 days old, *b* 5 days, *c* 3 days, *d* was fresh. Of these only *d* was effective in causing male *Nereis* to shed sperm. Each of the samples *a*, *b*, and *c*, had been tested previously at intervals and each was found gradually to lose its stimulating power in the course of about 3 days. As regards the heat-lability of the substance in question, a series of experiments have shown that it requires about 10 minutes boiling to destroy the power of an effective solution entirely. Five minutes boiling diminishes the effect considerably. The ineffectiveness after boiling is not due to the removal of oxygen, for it is impossible to restore its effectiveness by aerating.

The stimulating substance is either neutralized by the presence of sperm in the water owing to combination with some sperm

[1] See footnote, p. 150.

substance, or it ceases to be effective in the presence of a certain concentration of sperm. This is shown by the following experiment, June 20, 1911: A male put in about 8 c.c. of the water charged by a female immediately shed sperm which was stirred up by his movements; in a little while he stopped shedding. He was then transferred directly to another equal amount of the original charged water and the performance was repeated. A third transfer gave the same result, and on the fourth transfer a small amount of sperm only was shed, the male being practically spent by this time.

The same phenomenon was observed on other occasions. The explanation is, I believe, that the stimulating substance is a sperm agglutinin produced by the eggs, which enters into combination with the spermatozoa and thus disappears from the solution in the presence of a sufficient quantity of sperm. The reasons for this opinion are: (1) The sea-water charged by the female does, as a matter of fact, contain a sperm agglutinin which can be shown to be neutralized by the addition of a sufficient quantity of sperm. (2) This sperm agglutinin possesses about the same degree of heat lability as the substance effective in causing the sperm-shedding reaction of the male. The relations of the sperm to egg-secretions will form the topic of another paper, but they are summarized in a preliminary paper by the senior author in *Science*, N. S., Vol. XXXVI., p. 527. The conclusion to which we have come is, therefore, that the same egg-secretion of *Nereis* which agglutinates the spermatozoa is the effective stimulus in the sperm-shedding reflex of the male. This is the simplest explanation of the facts and may be held until some reason is shown for believing that different substances are involved. Such a condition is particularly well adapted to secure fertilization under the conditions of the breeding habits of *Nereis*.

II. Data on the "Runs" and the "Swarms."

By a "run" we mean an entire lunar cycle of the pelagic "*Heteronereis*"; by a "swarm" the nightly occurrence. A general statement of the facts was given in the introduction. The precise data with some comparisons follow:

1. *The Swarms.*—The swarms consist only of fully mature males and females; indeed we never secured even a single swarming animal which was not fully mature, or which contained any immature sexual elements mingled with the mature ones. The occurrence of swarming is dependent more upon the lunar cycle than any other factor; the relations with reference thereto are given under the second heading, "*The Runs.*" The second most important factor in swarming is time of day; swarming begins invariably soon after twilight and continues for an hour or two at the most. A third factor is weather; very bad weather with heavy wind and rain may prevent swarming, or at least prevent the animals from coming near enough to the surface to be seen. A fourth factor is light, partially included, of course, under the second factor. But we have reference here especially to conditions that occur at the time of full moon or a little later, when swarming may begin after twilight, and be suddenly cut short by the appearance of the moon above the eastern hills.[1] On the other hand during the light of the moon, *i. e.*, from shortly after new moon until near the time of the full moon the animals do not swarm even if the moon is entirely concealed by thick clouds.[2] The stage of the tide whether high or low was not an observable factor at our station, where the range of tidal movement is only about 18 inches. Neither do the summer variations in temperature appear to be an important factor; in any event the temperature of the water varies but little during the swarming season.

We have gained the impression that all the absolutely mature animals of any given locality swarm as soon as the right conditions are offered. This would involve the assumption that the maturing of the animals is dependent on some relation of the life history to the phases of the moon, involving probably,

[1] An exception to this was noted at the time of full moon in July, 1912, when after moonrise both males and females were abundant. (See curve 1, July 28 and 29.)

[2] As an exception it may be noted that the end of the June run (1912) extended to within three days of the full moon (see curve 6). But the general rule is as stated. Special attention was directed to this point both in 1911 and 1912. There were several cloudy nights during the light of the moon in these years about the time of the first quarter of the moon; for instance, on July 21, 1912, a hard rain fell in the afternoon and the evening was dark and cloudy, but no worms could be found. We often tried collecting in dark places under wharves, etc., on moonlit nights, but always without success.

through lunar tidal variations, rhythmical alterations of conditions of nutrition. (Cf. Hempelmann, 1911.) This assumption at least explains fairly well the conditions as we find them.

2. *The Runs.*—The collector receives very definite impression concerning the size and composition of swarms and the general features of the runs. But to give these quantitative expression is not a simple matter, though it is essential in order to reveal the order that exists. Moreover, any quantitative system of representing the data must be based on some one feature to the exclusion of others, and it is of course limited to this extent. As a result of experience we finally decided in the first place to limit the collections used in the tables to one station, and in the second place to record only females caught. The latter provision is due to the relatively enormous number of the males on certain nights which makes accurate estimation out of the question. The collecting was done each evening from the same station in the same way as nearly as possible. Practically complete records were kept for 1911 and 1912, and these have been tabulated in the form of curves in which the ordinates represent the number of females caught and the abscissæ represent days from the first quarter of the moon.

Of these records of runs we may select that of August, 1912, as being in some respects the most typical (see curve 1). It begins on the fifth day after the first quarter of the moon (July 25), and is quite atypical in this one respect, for no other run began earlier than the eighth day. On the fifth day two feamles were caught, the sixth day one, seventh day ten, eighth day eight, then the catch gradually ascends to a maximum on the twelfth day. The numbers in the successive swarms then rapidly diminish to the 15th day, when only three females were caught, but on the 16th day there were sixteen females, and on the 17th, 18th, 19th, 20th, and 21st, over thirty. The numbers then gradually decreased to the 26th day, which was the last day of the run. This run had been preceded by eight days in which no *Nereis* occurred, and it was followed by a barren period of eleven days It was therefore sharply separated from preceding and succeeding runs.

The other runs may be compared with this one as follows:

1. June, 1911; curve 2. The first collection was made on June 12, one day after the full moon or ten days after the first quarter. Judging by experience this was probably the second or third day of the run. That night 12 females were caught. The curve of this run is bimodal, sinking to zero on the 20th day from the first quarter, and then rising again. The last swarm of the run was on the 28th day after the first quarter. There is a depression in the first mode due to some unknown cause. This run was separated from the following July run by eleven days in which neither sex could be found.

2. The July, 1911, run (curve 3) began on the ninth day after the first quarter of the moon, thus on the night of the full moon (July 11). The curve of this run is also bimodal with a deep depression from the 17th to the 21st days; the last swarm was on the 26th day. Here also there is a depression in the first mode. There was an interval of eleven days from the last swarm of the July run to the first August swarm.

3. August, 1911. The run (curve 4) began on the eighth day after the first quarter and lasted to the 26th day. While the curve is decidedly irregular it is nevertheless distinctly bimodal, with a depression to zero on the 17th day. Seventeen days elapsed before the beginning of the September run.

4. September, 1911. The run (curve 5) began on the thirteenth day after the first quarter. The swarms were extremely small and few in number. But parts of the two modes of the usual curve may be recognized.

5. June, 1912; curve 6. The first worms were secured on the 13th day after the first quarter of the moon. I regard the collections from the 13th to the 17th days as constituting the first mode of the curve. The second mode is very unusual, first in its irregularity, and second in its duration; it continued in fact ten days after the new moon, and it exceeds all other records in this respect by six days. There was thus an interval of only four days between the end of June and the beginning of the July run in 1912. The last seven days of the June run thus occurred during the "light of the moon," a circumstance which must be regarded as significant. Swarming can thus take place during moonlight. Its usual absence must therefore be inter-

preted to mean that completely metamorphosed animals usually do not occur during this time.

6. July, 1912; curve 7. The run began on the ninth day after the first quarter of the moon, on the night of the full moon (June 29). This run shows no evidence of two modes, for the lack of animals on the second night of the run cannot be interpreted as the intermodal depression. The run continued until the 27th day. The absence of two modes in this case may be attributed to overlapping of the two sub-runs making up each run. This run was followed by eight days without any swarms. The August, 1912, run, already described, finished on the 26th day after the first quarter; and there was an interval of eleven days before the beginning of the September run.

7. September, 1912; curve 8. This run began on the eighth day after the first quarter of the moon, and ended unusually early on the 23d day. Though the curve is irregular in its first part there is some evidence of bimodality.

The two features that stand out most prominently in these records are the relation of the runs to the lunar cycles, and the double character of each run. The following tabulation brings out the first point; the days being numbered from the first quarter of the moon in each case:

1911. First run, tenth to twenty-eighth day.
Second run, ninth to twenty-sixth day.
Third run, eighth to twenty-sixth day.
Fourth run, thirteenth to twenty-first day.

1912. First run, thirteenth to thirty-fourth day.
Second run, ninth to twenty-seventh day.
Third run, fifth to twenty-sixth day.
Fourth run, eighth to twenty-third day.

As regards the second point, the curves show sufficiently well that each run is made up of two sub-runs. But they are not usually sharply separated by an interval as in the case of the main runs; however, the general rule is that the interval occurs near the time of the third quarter of the moon. Thus the low points in the runs as tabulated are:

1911.	Days after First Quarter.
First run	20
Second run	17–21
Third run	17
Fourth run	15–21

1912.	
First run	16–17
Second run	?
Third run	15
Fourth run	15

The third quarter of the moon comes 16–17 days after the first quarter.

Hempelmann (1911) finds that the swarms of *Nereis dumerilii* at Naples tend to center around the time of the first and third quarters of the moon; none were caught on the days of the new moon or the full moon. Thus, the monthly curve is bimodal as in the case of *Nereis limbata*, but the modes, occurring at the time of the first and third quarters, do not coincide, but alternate with those of *N. limbata* which approximate the times of the full moon and the new moon. The animals were secured from the daily plankton catch taken in the early morning hours, so little is known about the duration of the swarming or the time of its maximum. But the indications are that the time of the daily swarm is early morning (before sunrise?), and this furnishes another contrast to *N. limbata*. The runs of *N. dumerilii* occurred at Naples from October to May.

According to Akira Izuka (1903) the Japanese palolo (*Ceratocephale Osawai*) swarms closely following the new moon and the full moon. The swarms occur shortly after sunset and last an hour and a half to two hours. Each run is limited to three or four nights in the months of October and November. Thus there are two entirely distinct runs each month in this case corresponding to the sub-runs in the case of *Nereis limbata*. About ten days after each swarming period transitional half-epitokous forms are found in the bottom mud, which finish their metamorphosis in time for the next swarming period, after which only immature animals are to be found in the mud. The periodic swarming is thus dependent on periodic maturation of the animals. In many respects the swarming of *Ceratocephale* is more like that of the Woods Hole *Nereis* than any other form.

The breeding habits of the Pacific palolo (*Eunice viridis*), the Atlantic palolo (*Eunice furcata*), and *Odontosyllis* (Galloway and Welch, 1911), are similar with respect to periodicity dependent on lunar phases, though of course different in many details.

Some other annelids (*e. g., Amphitrite;* see Scott, 1909) exhibit a lunar periodicity in breeding habits. The periodicity in *Nereis limbata* thus belongs to a large class of phenomena, which includes breeding seasons of some plants as well as many other animals, for which no adequate general cause has yet been found.

III. The Life History of *Nereis limbata.*

This subject is still under investigation. It is obvious that interpretation of the swarming behavior must wait for fairly complete knowledge of the life history. The present indications are that the worms either do not mature in the atokous condition, or, if they do so, that it is at an earlier time of year than our collections were made. The atokous forms were secured both in scrapings from piles of wharves and also by digging, and were kept under observation in the laboratory. All that matured underwent metamorphosis to the epitokous condition. Despite repeated efforts we failed to rear mature worms from artificially fertilized eggs. However, a few were raised to young worms about 5 mm. in length, about eight weeks after fertilization; but all of them died after an attempted change in culture methods.

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General Description of Curves.

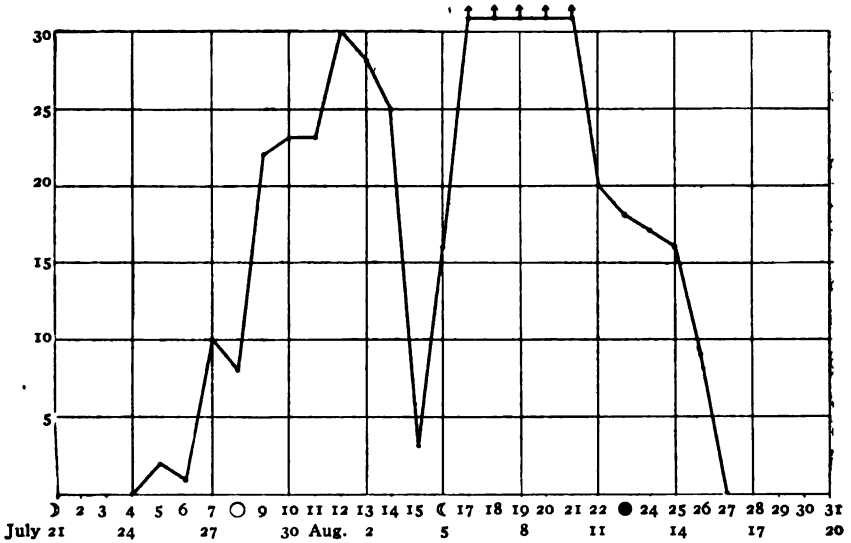
The ordinates represent the number of females caught.

The abscissæ represent days from the first quarter of the moon. The various phases of the moon are indicated. The days of the month are given in the second horizontal row of figures below the curves.

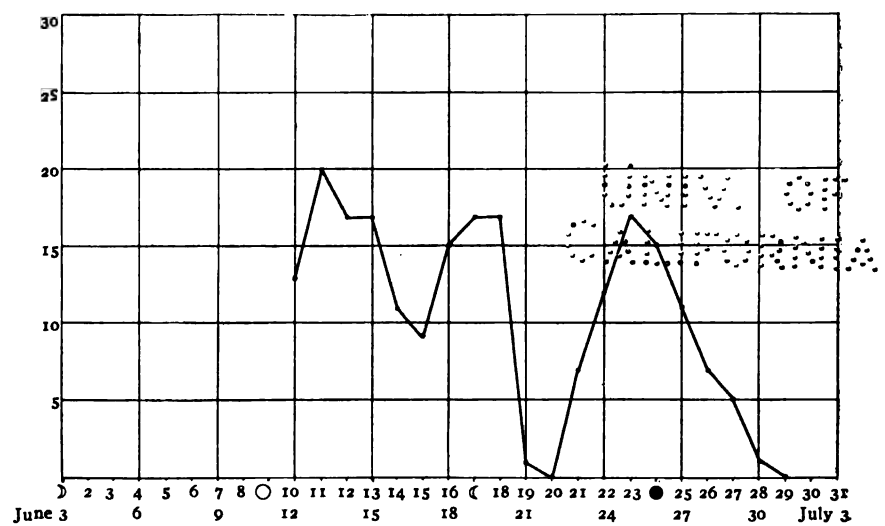
Curve 1. The August run, 1912.

Curve 2. The June run, 1911.

CURVE 1



CURVE 2



CURVE 3. The July run, 1911.
CURVE 4. The August run, 1911.

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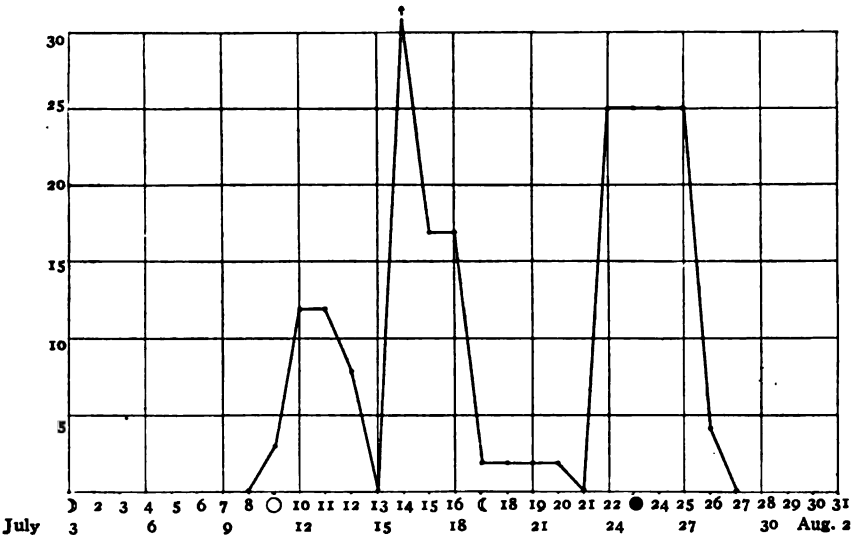
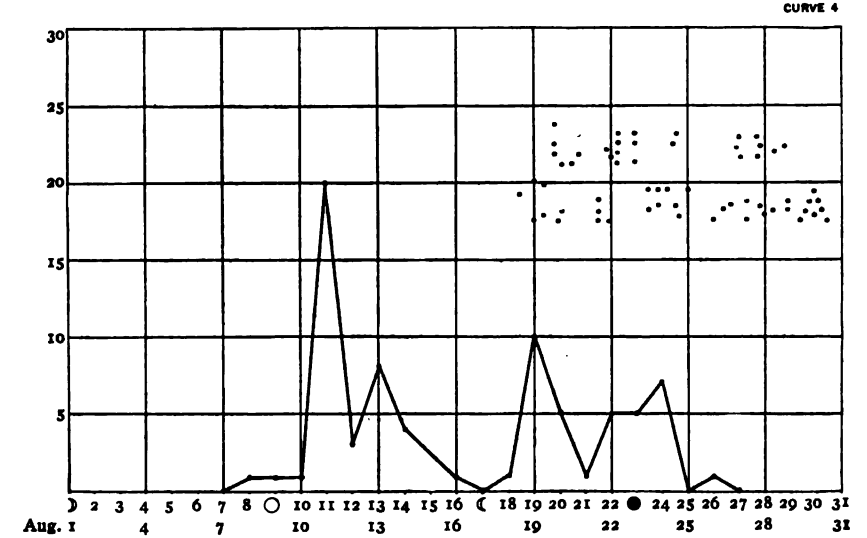
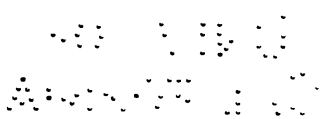
CURVE 3



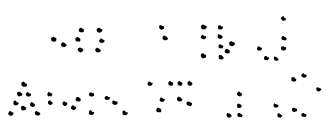
CURVE 4

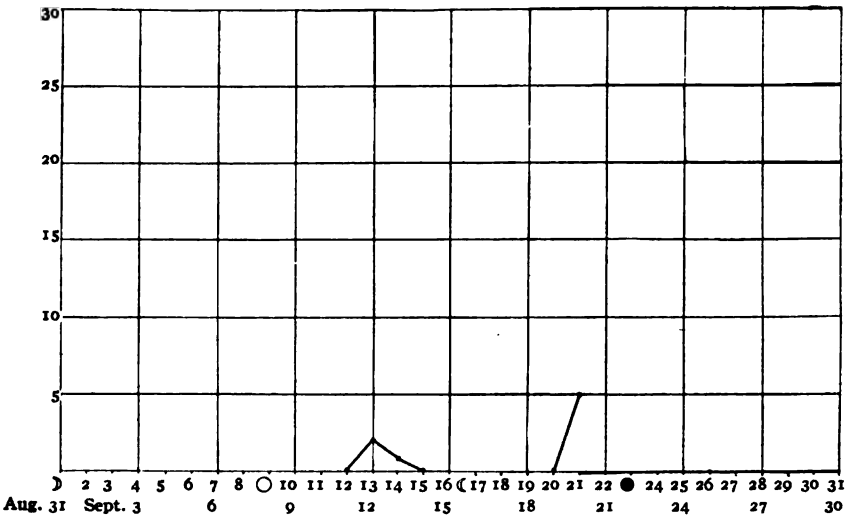




CURVE 5. The September run, 1911.

CURVE 6. The June run, 1912. The part of the curve to the left is the end of the June run as indicated by the arrow at the extreme right, and by the second row of dates which refer to this part of the curve (see footnote, p. 154).

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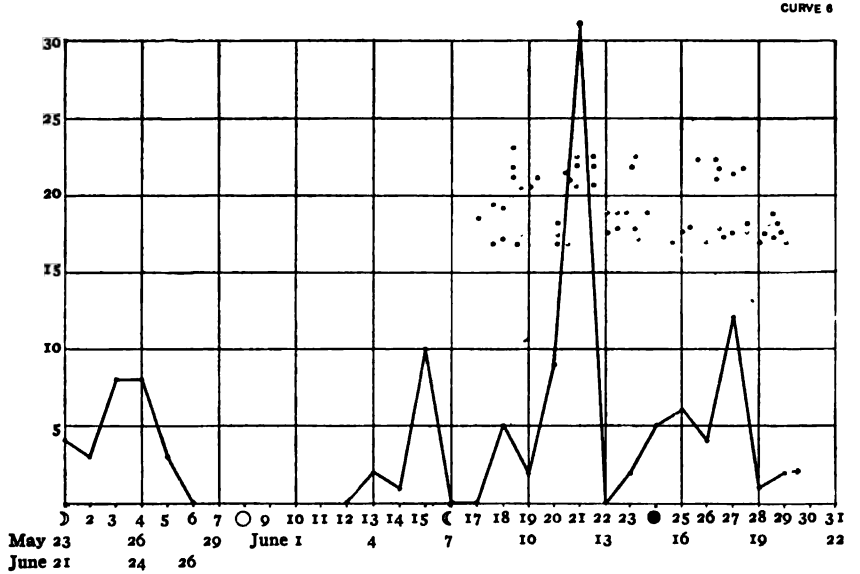
CURVE 6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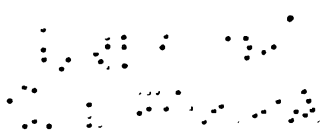
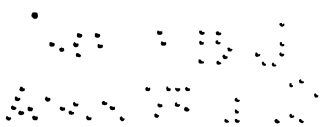
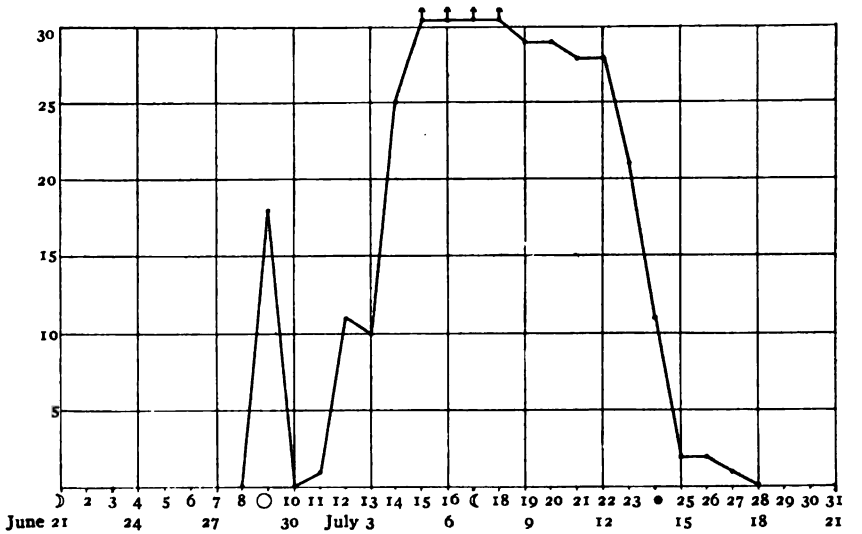
June 21 24 26



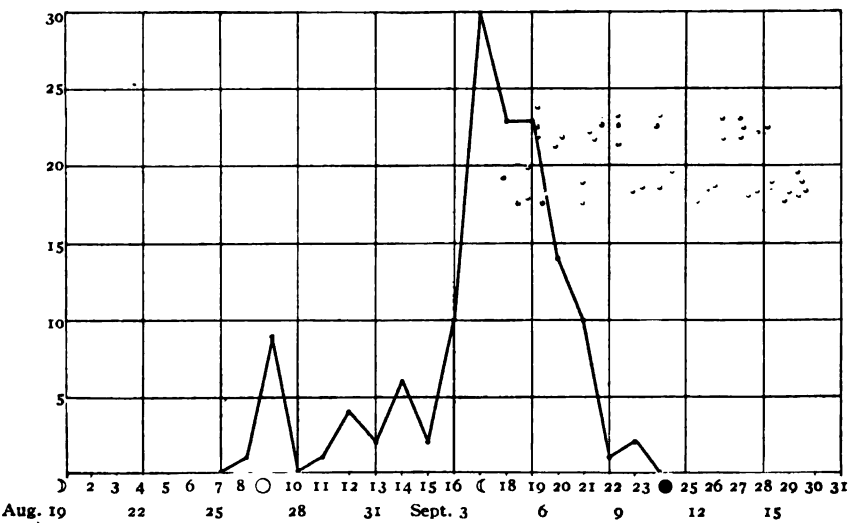
CURVE 7. The July run, 1912.
CURVE 8. The September run, 1912.

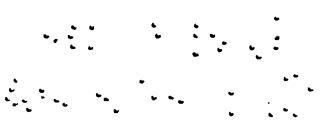


CURVE 7



CURVE 8





[*Reprinted from* SCIENCE, *N. S., Vol. XXXVIII., No. 980, Pages 524–528, October 10, 1913*]

THE MECHANISM OF FERTILIZATION

IN previous papers[1] I have described the secretion of a substance by the ova of the sea-urchin, *Arbacia*, in sea water, which causes agglutination of the sperm of the same species. The eggs of *Nereis* also secrete a substance having a similar effect upon its sperm. I therefore named these substances sperm-iso-agglutinins. During the present summer I have ascertained that in the case of *Arbacia*, and presumably also of *Nereis*, the agglutinating substance is a necessary link in the fertilization process and that it acts in the manner of an amboceptor, having one side-chain for certain receptors in the sperm and another for certain receptors in the egg. As this substance represents, presumably, a new class of substances, analogous in some respects to cytolysins, and as the term agglutinin defines only its action on sperm suspensions, I have decided to name it fertilizin.

My main purpose this summer was to study the rôle of the *Arbacia* fertilizin in the fertilization of the ovum.

1. *The Spermophile Side-chain.*—The first need in such a study was to develop a quantitative method of investigation, and this was done for *Arbacia* as follows: The agglutinative reaction of the sperm in the presence of this substance is, as noted in previous studies, reversible, and the intensity and duration of the reaction is a factor of concentration of the substance. The entire reaction is so

[1] SCIENCE, N. S., Vol. 36, pp. 527–530, October, 1912, and *Journ. Exp. Zool.*, Vol. 14, No. 4, pp. 515–574, May, 1913.

characteristic that it was possible to arrive at a unit by noting the dilution at which the least unmistakable reaction was given. This was fixed at about a five- or six-second reaction, which is counted from the time that agglutination becomes visible under a magnification of about 40 diameters until its complete reversal. The unit is so chosen that a half dilution gives no agglutination of a fresh 1 per cent. sperm suspension. It was then found that the filtrate from a suspension of 1 part eggs left for ten minutes in 2 or 3 parts sea water would stand a dilution of from 800 to 6,400 times, depending on the proportion of ripe eggs and their condition, and still give the unit reaction. Such solutions may then be rated as 800 to 6,400 agglutinating power, and it is possible, therefore, to determine the strength of any given solution. This gives us a means of determining the rate at which eggs are producing fertilizin in sea water.

Determinations with this end in view showed that the production of fertilizin by unfertilized eggs of *Arbacia* in sea water goes on for about three days and that the quantity produced as measured by dilution tests diminishes very slowly. Such tests are made by suspending a given quantity of eggs in a measured amount of sea water in a graduated tube; the eggs are then allowed to settle and the supernatant fluid poured off and kept for testing. The same amount of fresh sea water is then added and the eggs stirred up in it, allowed to settle, the supernatant fluid poured off for testing, and so on. In one series running three days in which the quantity of eggs was originally 2 c.c. and the total volume of sea water and eggs in the tube 10 c.c., 6 to 8 c.c. being poured off at each settling, thirty-four changes were made and the agglutinating strength of the supernatant fluid diminished from 100 at first to 20 at the end. Simultaneously, with this loss of agglutinating strength,

two things happen: (1) the jelly surrounding the eggs undergoes a gradual solution; (2) the power of being fertilized is gradually lost.

It is obvious that the presence of fertilizin in such considerable quantities in so long a series of washings shows either (1) that solution of the jelly liberates fertilizin, or else (2) that the eggs secrete more fertilizin each time they are washed. Both factors enter into the case inasmuch as (1) eggs killed by heat (60° C.) will stand 14 or 15 such washings, but with more rapid decline of agglutinating power than the living eggs. The jelly is gradually dissolved away in this case also, and is presumably the only possible source of the agglutinating substance. (2) Eggs deprived of jelly by shaking continue to produce the fertilizin as long as eggs with jelly, though in smaller quantities at first, and they are equally capable of fertilization.

Not Complete.

The fertilizin is therefore present in large quantities in the jelly, which is indeed saturated with the substance, but the eggs continue to produce it as long as they remain alive and unfertilized. When the eggs are fertilized the production of this substance suddenly ceases absolutely.

The total disappearance of fertilizin from fertilized eggs can not be demonstrated unless the fertilizin-saturated jelly with which the eggs are surrounded be first removed. This is very easily done after membrane formation by six vigorous shakes of the eggs in a half-filled test tube. Three or four washings then are sufficient to remove the remains of the jelly, and the naked eggs no longer produce the substance.

Such disappearance may be due either to complete discharge from the egg, or to fixation of all that remains by union with some substance contained in the egg itself. That such a substance—anti-fertilizin—exists in the egg can be shown by a simple test-tube experi-

ment: If eggs deprived of jelly are washed 34 times in sea water during three days, they are so exhausted that they produce but little fertilizin; the supernatant fluid may be charged only to the extent of 2 to 10 units. The eggs are now on the point of breaking up. If they are then vigorously shaken and broken up so that the fluid becomes colored with the red pigment of the eggs, it will be found that agglutinating power has entirely disappeared from the solution. The fertilizin present has been neutralized. The same phenomenon may be demonstrated also by treating eggs, deprived of jelly in order to get rid of excess of fertilizin, with distilled water which lakes the eggs and extracts the anti-fertilizin.

It is probable, therefore, that any excess of fertilizin remaining in the egg not bound to the sperm is neutralized by this combination, and polyspermy is thereby prevented.

We have noted (1) the secretion by unfertilized eggs in sea water of a sperm agglutinating substance, fertilizin; (2) the extreme avidity of the sperm for it as shown by dilution tests; (3) in my previous papers the fixation of this substance in sperm-suspensions of the same species (quantitative measurements will be given in the complete paper); (4) the sudden cessation of fertilizin production by fertilized eggs; (5) the existence of an anti-fertilizin in the egg; (6) in eggs submitted to a series of washings decrease of the fertilization capacity with reduction of the fertilizin. The fact that fertilized eggs can not be refertilized is associated with the absence of free fertilizin in them; (7) I may add that, similarly, eggs in which membrane formation has been induced by butyric acid can not be fertilized by sperm and they contain no free fertilizin.

It is therefore very probable that the substance in question is essential for fertilization.

It may be maintained that these facts do not constitute demonstrative evidence of the necessity of this substance for fertilization, for the presence or absence or diminution of this material associated with presence or absence or decrease of fertilizing power could always be regarded as a secondary phenomenon. However, the second part of this paper dealing with the other, or ovophile side-chain of the fertilizin, strongly reinforces the argument.

Before passing on to this, I may be allowed to note some other properties of the fertilizin: In my previous papers I noted the extreme heat-resistance of the fertilizin, being only slowly destroyed at 95° C. I also noted that strongly agglutinating solutions of *Arbacia* may contain a substance which agglutinates *Nereis* sperm and stated that this was probably different from the iso-agglutinating substance. This turns out to be the case and the two can be readily separated. The substance must possess great molecular size, as it is incapable of passing through a Berkefeld filter. It is also non-dialyzable; it does not give the usual protein reactions, a fact for the determination of which I am indebted to Dr. Otto Glaser.

2. *The Ovophile Side-chain.*—Assuming, then, that the union of this substance with the spermatozoon enters in some significant way into the process of fertilization, the problem was to ascertain in what way. The simplest idea, viz., that the union is in itself the fertilization process, was soon shown to be untenable, for the reason that the perivisceral fluid (blood) of the sea-urchin, especially of ripe males and females, often contains a substance which absolutely inhibits fertilization in the presence of any quantity of sperm, but that this substance has no inhibiting effect at all upon the sperm-agglutination reaction. It

does not enter into combination with the spermophile side-chain. In other words, the binding of the agglutinin by the sperm may be complete, but in the presence of an inhibitor contained in the blood none of the usual effects of insemination, no matter how heavy, follow.

The details of the experiments upon which the above statement depends are too complex for consideration here. But they showed that the effect is neither upon the egg alone nor upon the sperm alone, for both may stand for some time in the presence of this agent and after washing be capable of normal behavior in fertilization, though there may be some decrease in the percentages. No poisonous effect is involved on either sexual element.

The next suggestion was fairly obvious, viz., that the substance which we had been calling agglutinin, on account of its effect upon the spermatozoa, is in reality an amboceptor with spermophile and ovophile side-chains, and that the binding of the sperm activates the ovophile side-chains which then seize upon egg receptors and fertilize the egg. If this were so, it is obvious that the spermatozoon is only secondarily a fertilizing agent, in the sense of initiating development, and that the egg is in reality self-fertilizing, an idea which agrees very well with the facts of parthenogenesis and the amazing multiplicity of means by which parthenogenesis may be effected. For the agents need only remove obstacles to the union of the amboceptor and egg receptor.

The inhibiting action of the blood from this point of view is a deviation effect due to occupancy of the ovophile side-chain of the amboceptor, either because the inhibitor in the blood is an anti-body to the amboceptor or because it possesses the same combining group as the egg receptor. In such a case, the ovophile group of the amboceptor, being already

occupied by the inhibitor, fertilization could not take place.

Fortunately, this idea is susceptible of a ready test; for, if the blood acts in this way in inhibiting fertilization, all that is necessary to neutralize the inhibiting action would be to occupy the inhibitor by the amboceptor (fertilizin) for which *ex. hyp.* it has strong affinity. This experiment was repeated many times in different ways with various dilutions, and the result was always to lessen or completely remove the inhibiting action of the blood.

The plan of such an experiment is this: to divide the filtered blood (plasma) in two parts, one of which is used for control while the other is saturated with fertilizin by addition of eggs. In ten minutes the latter are precipitated by the centrifuge and the supernatant fluid filtered. Fertilizations are then made in graded dilutions of this and the control blood. In some cases the inhibiting action of the blood was completely neutralized, and in all largely neutralized.

The results so far are in agreement with the theory. But if it be true that the egg contains its own fertilizing substance, it might also be possible to induce parthenogenesis by increasing the concentration of this substance to a certain point; though it is conceivable that no increase in concentration would break down the resistance that normally exists to union of the amboceptor and egg receptors. As a matter of fact, Dr. Otto Glaser[2] has shown this summer that a certain amount of parthenogenetic action may be induced in *Arbacia* in this way. I have been in consultation with Dr. Glaser during part of his work and can confirm his statements.

In connection with the assumption that the sperm activates an already existing side-chain

[2] SCIENCE, N. S., Vol. XXXVIII., No. 978, September 26, 1913, p. 446.

of a substance contained in the egg itself, I may be allowed to cite the following statement of Ehrlich:

> The significance of the variations in affinity will be discussed connectedly at a subsequent time. We shall content ourselves here by pointing out that an understanding of the phenomena of immunity is impossible without the assumption that certain haptophore groups become increased or decreased in their chemical energy, owing to changes in the total molecule. Chemically, such an assumption is a matter of course.[8]

This principle might explain the activation of the fertilizing amboceptor by the sperm.

The question will of course be raised whether there is not another and simpler interpretation of the facts. There are three general classes of these facts: (1) the sperm agglutination phenomena, and the apparent necessity of the agglutinating substance for fertilization; (2) the presence of an inhibiting agent in the blood, especially of ripe males and females; (3) the neutralization of this inhibiting agent by the agglutinating agent (amboceptor). It may be questioned whether these facts have the particular causal nexus that I have given them. But I think it would be difficult to construct a theory taking account of all the facts which would differ essentially from that presented here.

The theory is really extremely simple in its character, and the facts on which it rests are readily tested. It has proven a most valuable working hypothesis; indeed, many of the facts referred to were discovered only after the theory was formed. It has the advantage of offering one theory for initiation of development whether by fertilization or by parthenogenesis. It is capable of explaining the whole range of specificities in fertilization by assuming a specific fertilizin for each species. It furnishes the foundation for the chemical

[8] "Collected Studies in Immunity," p. 220.

conceptions necessary to any theory of fertilization, and it is susceptible of experimental test.

It will be seen that inhibition of fertilization may occur by block in any part of the mechanism.

1. Through loss of fertilizin by the egg.
2. Through occupancy of the sperm receptors.
3. Through occupancy of the egg receptors.
4. Through occupancy of the ovophile side-chain of the amboceptor (fertilizin).
5. Through occupancy of the spermophile side-chain group.

Of these I have shown the occurrence of the first, fourth and fifth in *Arbacia*. The first in the case of long-washed eggs; the fourth in the case of the inhibitor contained in the blood; the fifth is, I believe, the mechanism for prevention of polyspermy.

The mechanism of fertilization appears to be the same in *Nereis*, though I have not a complete set of data. However, the data that I have are in accord with the theory, and will be described in the complete paper.

I should perhaps state specifically that the location of the fertilizin is in the cortex of the egg.

It seems to me probable that the activation of the fertilizin is by no means confined to that bound by the single penetrating sperm, but that activation once set up spreads around the cortex. The supernumerary spermatozoa that fail to enter the egg may also play a part by setting up centers of activation. In this connection Glaser's contention that several spermatozoa at least are necessary for fertilization is of great interest. The nature of the effect of the activated fertilizin on the egg is analogous in some respects to a superficial cytolysis, in this respect agreeing with Loeb's theory. But the "lysin" is contained in the egg, not in the sperm, as Loeb thought;

if cytolysis is involved, it is a case of autocytolysis. This may involve increase of permeability, the effects of which R. S. Lillie has especially studied. I mention these possibilities in order to point out that the conception contained in this paper is not in conflict with the well-established work of others.

In conclusion, I may point out that the theory assumes a form of linkage of sperm and egg components by means of an intermediate body that may find a place in the study of heredity. The detailed experiments will be published later.

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WOODS HOLE, MASS.,
September 1, 1913

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SPERM AGGLUTINATION AND FERTILIZATION.

FRANK R. LILLIE.

In a recent paper on "Cluster Formation of Spermatozoa Caused by Specific Substances From Eggs" Loeb ('14) has presented a criticism of my theory of fertilization (Lillie '13*b* and '14), based on observation of the California sea-urchin *Strongylocentrotus purpuratus*. My own observations were made on *Arbacia punctulata* of Massachusetts, and it would appear that part at least of Loeb's criticism was due to certain differences in the two forms, for he has now stated (Loeb, 1914*b*, p. 318, footnote) that the "cluster formation" of the spermatozoa may find its explanation "on the assumption of an agglutination at least in the case of *Arbacia*," as I maintained; it is therefore not a "tropistic reaction" as he thought probable from his observations in California. This was one of the chief differences of opinion. A second one was in regard to the source and significance of the substance in the fluid of egg suspensions that caused such agglutination; Loeb maintained that it was merely the dissolved chorion (*i. e.*, jelly layer) of the egg, and that after this was removed the eggs no longer produced the agglutinating substance, and yet were capable of fertilization; whereas my contention was that the agglutinating substance was a secretion of the egg soaked up by the jelly, as by a sponge; that the eggs produced it for a certain length of time after the removal of the jelly, and lost their power of fertilization after they ceased to produce it.

These criticisms cut at the foundation of my theory. Inasmuch as the correction of the tropistic interpretation of agglutination is given only in a footnote to another paper, and no correction of the source of the agglutinating substance has yet appeared, it is incumbent on me to consider the criticisms carefully; at the same time I wish to take the opportunity to explain certain points that appear to be open to misinterpretation, and to record some new observations.

1. "Cluster-formation" versus Agglutination.

The phenomena exhibited by sperm suspensions of *Arbacia* with which we have to deal are of four distinct types, which it is essential to distinguish sharply: (1) activation; (2) aggregation; (3) agglutination, (4) mass-coagulation.[1] (1) That the activity of spermatozoa is affected by substances in the sea-water requires no argument. The subject is discussed in study V (Lillie, 1913*a*, pp. 519–532). (2) Aggregation of spermatozoa may be brought about by tropistic reactions. In my paper on the "Behavior of Spermatozoa," I devoted a great deal of attention to such aggregation phenomena and the distinction from phenomena of agglutination (1913, pp. 532–548 and pp. 551–552). Among other things I pointed out that aggregation as a tropistic phenomenon implies a gradient,[2] and that the spermatozoa never adhere, however crowded they may be; there is no observable physical change of the spermatozoa and the slightest agitation suffices to disperse them again. Such tropistic phenomena may be exhibited in response to CO_2 and other acids (*Nereis*), or certain constituents of egg secretions, to mention only chemotaxis.

(3) Agglutination of spermatozoa on the other hand requires no gradient, and the spermatozoa adhere physically to such an extent that the agglutinated masses may be preserved intact in killing fluids; its degree is a function of the concentration of the agglutinating medium, and is also different in different species. Agglutination is non-toxic, not limiting the life of the spermatozoa; it is reversible, its duration depending on the concentration of the agglutinating medium; it cannot be repeated if the reaction is complete, at least within the time limits of my experiments, even though the spermatozoa remain motile;[3] finally motility of the spermatozoa is a prerequisite to a decided reac-

[1] The reaction here referred to is a lethal phenomenon. It possibly involves cytolysis with subsequent adhesion of the spermatozoa.

[2] It is important to notice that the spermatozoa of suspensions may produce gradients through their own activities. Thus I pointed out that autogenous aggregation reactions in sperm suspensions of *Nereis* arise from the positive chemotaxis of the spermatozoa to their excreted CO_2, giving rise to very striking phenomena (Lillie, 1913*a*, pp. 519–521 and pp. 538–540). It is conceivable that such a tropistic phenomena is involved as a part factor in the agglutination phenomena under discussion.

[3] Glaser (1914) also comes to this conclusion.

tion; evidently because the physical change on which the reaction depends is not sufficient to cause adhesion except when the spermatozoa positively collide.[1] These six criteria definitely define the phenomenon.

Agglutination is positively distinct from aggregation. It is an entirely different biological phenomenon. The two may, however, be exhibited simultaneously, as when a drop of egg secretion of *Arbacia* is injected into a sperm suspension of the species. In such a case the spermatozoa exhibit positive chemotaxis to one constituent of the egg secretion, and are agglutinated by another (the fertilizin). The separateness of these two substances was maintained in my first publication on the subject and demonstrated by repeated experiments (see Lillie, 1913*a*, p. 549, and 1914, pp. 545–546).

(4) The phenomenon of mass-coagulation is, on the other hand, a lethal irreversible phenomenon. It may be exhibited in response to various agents, such as KOH, NaOH, salts of lanthanum and cerium,[2] etc., and in some cases the secretions of the eggs of other species or their blood. Hitherto I have not adequately defined this phenomenon as distinct from the agglutination phenomena, though in my last study (1914), I noted the distinction (p. 541). The phenomenon is essentially lethal, but not all destructive agents exhibit it; thus acids, so far as I have observed, destroy the spermatozoa without causing mass coagulation. The phenomenon is irreversible, and this suffices to distinguish it from true agglutination, even if no other criterion were available. However, it exhibits quite a different aspect from agglutination; in the latter the sperm masses tend to take on a spherical form; if originally elongated they contract into balls or break up into smaller masses which become spherical, thus offering considerable resemblance to a phenomenon of surface tension, as Loeb notes. The peripheral spermatozoa are in violent movement until the time of reversal. In the mass-coagulation reaction, on the other hand, there is no such surface tension effect, strands anastomose

[1] Loeb argues that the necessity of movement on the part of the spermatozoa for the appearance of this phenomenon removes it from the category of true agglutination; but this seems to me to be a purely arbitrary criterion.

[2] My attention was called to the action of the salts of these metals by a letter from James Gray of Cambridge University.

with other strands and form a net-work and the movements of the spermatozoa soon cease.

The substances of egg secretions, which I have hitherto called hetero-agglutinins, belong to this category, in some cases at any rate. Though I will not assert that there is no such substance as a hetero-agglutinin in the real sense of agglutination, yet the substance in *Arbacia* blood, or egg secretions, the effects of which on *Nereis* sperm I have previously studied, should be regarded as a toxic rather than an agglutinating substance, having the mass coagulant action. As I stated in my last paper, p. 541, it produces a permanent coagulum in *Nereis* sperm suspensions; "in this respect the action differs from the iso-agglutination, which is without toxic effects."

We must keep firmly in mind the distinctions between aggregation (tropisms) agglutination, and mass-coagulation. Agglutination, with which we are particularly concerned, is distinguished from aggregation by the facts that it occurs in the absence of a gradient, it involves physical adhesion, and cannot be repeated if the reaction is once complete; it also is characterized by a high degree of specificity.[1] From mass coagulation it is distinguished by the facts (1) that it is non-toxic, (2) reversible, (3) dependent on motility of the spermatozoa. Agglutination occurs so far as I have observed with certainty only in response to egg-secretions of the same species.

For description of the phenomena of agglutination of sperm by egg-extractives of the same species, I must refer to my previous paper (Lillie, 1913*a*); the phenomenon in *Arbacia* is a true agglutination in the sense defined, not a tropistic reaction, nor yet a mass coagulation. Loeb has admitted this for *Arbacia*, and I would therefore venture to suggest the probability that the phenomenon which Loeb has described in *Strongylocentrotus* and termed "cluster formation," which he interprets with some reserve as a possible tropistic reaction, is also true agglutination, which differs only quantitatively from *Arbacia* and *Nereis*. The

[1] Loeb admits that the "cluster formation" exhibits a high degree of specificity. It is therefore inconsistent to interpret the reaction, as he also does, as a "possible tropistic phenomenon" because such phenomena so far as we know do not exhibit specificities of this kind. Agglutination phenomena, on the other hand, as is well known, commonly exhibit equal specificity of a similar kind.

conditions under which it occurs, in response to egg secretions of the same species, its character, reversibility, and the specificity of the reaction are identical with *Arbacia*. It is apparently, however, less pronounced, and therefore not so readily recognizable of itself as an agglutination phenomenon. Even the "apparent surface tension phenomena" which Loeb describes for the clusters—"Short streaks or cylinders contract into spherical masses, the above described clusters; and long cylinders break up into a series of small clusters"—are the same as I previously described for *Arbacia* (1913*a*, pp. 550–551).

Loeb's interpretation of the "cluster-formation" as a possible tropistic reaction confuses the two sets of phenomena—viz., aggregation (a true tropistic phenomenon) and agglutination—which sperm suspensions may exhibit to the egg-sea-water of its own species. But the aggregation (tropism) can take place only when there is a gradient from the secretion to the spermatozoa. This is realized under the conditions of my experiment of injecting a drop of egg-sea-water into a fresh sperm suspension beneath a raised cover slip; in such a case the two phenomena take place simultaneously viz.; aggregation in the form of a ring around or in the introduced drop (depending on concentration), and agglutination. These two phenomena are produced by two constituents of the egg-sea-water, as I have already maintained.

For the study of the aggregation phenomena therefore it is desirable to employ an agent which has no agglutinative action. This I did in an extensive series of experiments by the method just referred to (1913, p. 533 ff.). To illustrate:—a drop of a $^1/_{100}$ dilution of a saturated solution of CO_2 in sea-water injected into a sperm suspension of *Nereis* in sea-water mounted beneath a raised cover-slip is marked within a few seconds by the formation of a ring of active spermatozoa within the margin of the introduced drop, and separated from the general sperm suspension by a clear zone nearly free of spermatozoa 1.5 to 2 mm. in diameter. I interpreted the ring formation as a positive reaction to the attractive substance (CO_2 and acids generally); the spermatozoa follow the gradient from the suspension into the drop containing CO_2 a certain distance, *i. e.*, up to a certain concentration, and

are there arrested. The proof of this interpretation is found in the fact that, if increasing concentrations of CO_2 are used, the ring forms outside the drop and becomes progressively wider, *i. e.*, the migration ceases at a distance from the center which increases with CO_2 or acid concentration (see 1913*a*, pp. 536–538). Loeb suggests that the ring formation with a clear external zone around it is "an indication that the spermatozoa are negatively chemotropic to the strong egg-sea-water, and possibly positively chemotropic to the more diluted egg-sea-water, or to the dense collection of spermatozoa in the ring." The latter suggestion is of course untenable as a primary cause, for the ring-formation is precisely the phenomenon to be explained. It is also unnecessary to assume any negative tropism; the ring formation is due to a limitation of the positive movement by concentration. This is fully discussed in the paper referred to above, but Loeb does not allude to the discussion.

2. The Source of the Agglutinating Substance.

Professor Loeb has also taken issue with me on the question of the origin of the agglutinating substance. He regards his experiments as proving that the substance which causes the "cluster formation" is not formed in the egg but in the chorion; *i. e.*, in the layer of jelly which surrounds the egg. On the other hand I regarded it (and still hold to the opinion) as a secretion of the egg; with which the jelly of course becomes saturated.[1] Loeb's observations again were on *Strongylocentrotus* and mine on *Arbacia*. The issue is a real one even though the chorion is itself a secretion of the egg in earlier stages.

Loeb's conclusion was based on his observation that if the chorion be dissolved off in dilute hydrochloric acid in sea-water, the naked eggs transferred to sea-water produce no detectable amounts of the agglutinating substance any more, whereas the acid sea-water contains it in large quantities. My conclusions were based on the observation that when eggs of *Arbacia* are deprived of jelly (chorion) by shaking, or a prolonged series of

[1] Glaser (1914) also agrees substantially with me: "the agglutinating substance is located in greatest abundance in the jelly and the eggs also contain this material," p. 371.

washings, they still continue to produce the agglutinating substance in sea-water, though in much diminished quantity; in my full paper, which Loeb had not the opportunity of consulting, I gave series of measurements on this point (1914, pp. 532–538); I also pointed out that in immature ovaries containing many primary ovocytes, but some mature eggs, the quantity of agglutinating substance produced was relatively very small (1914, p. 530), and I therefore suggested that the substance was secreted by the eggs at the time of maturation and was soaked up by the jelly as by a sponge. The eggs, however, continue to produce it after maturation, as I shall show. The immature eggs have as thick a chorion as the mature eggs; therefore the agglutinating substance cannot be merely dissolved chorion. I recognized the possibility of the view expressed by Loeb, investigated it as fully as possible at the time, and rejected it.

Since Loeb's paper has appeared, I have repeated his experiments and found my former observations and conclusions confirmed in all respects:

Experiments.—The optimum concentration of HCl for removal of jelly without injury to eggs was found to be 50 c.c. sea-water + 1.4 c.c. N/10 HCl. 1.2 c.c. N/10 HCl in 50 c.c. sea-water did not fully remove the jelly, and 1.6 c.c. caused too much injury to the eggs evidenced by heavy agglutination and later cytolysis. In an experiment of July 17, 1914, the three above concentrations were used. The complete removal of the chorion in the intermediate concentration was demonstrated by observation of the eggs in a thick suspension of India ink in sea-water; even the minutest traces of adherent jelly can readily be detected by this method, but it was all gone. The eggs were then washed as follows: 10.11 A.M. 42/6 c.c.; 10.40 51/5 c.c.; 10.58 50/4 c.c. The supernatant fluid was then tested and found to be free from sperm agglutinating substance; thus furnishing proof that all originally contained in the jelly had been washed out. At 11.20 the supernatant fluid was poured off leaving only 5 c.c. in the tube. The eggs were allowed to settle, and at 11.25 the supernatant fluid was tested and gave a 9–10-second agglutination reaction with fresh sperm suspension. Thus these eggs entirely deprived of jelly by HCl are producing agglutinating substance.

At 4.25 P.M. the eggs were washed again 5/0.7 c.c. and the new fluid gave a 14-second reaction. The next morning the same eggs were washed again 5.5/1 c.c. The new fluid gave a 6–7-second reaction.

These results may be expressed in a different way: thus in an experiment of July 20, a series of eight successive washings of eggs deprived of jelly by acid sea-water represented a dilution of the agglutinating substance contained in the acid sea-water remaining with the eggs of 12,700,800 times. But the acid solvent itself was negative at 1/800 dilution: it was of 400 agglutinating power. In other words, after the removal of the jelly the eggs themselves had produced a sufficient quantity of the agglutinating substance to account for the tremendous difference; and they were still producing it.

These eggs without jelly are fertilizable, as Loeb states, but only 37 per cent. segmented in a heavy insemination of the first day in the experiment of July 17, and only a small part of these developed to the ciliated stage, none of which were normal, most being stereoblastulae and incapable of farther development. The result is entirely similar to that described in my last paper (study VI, '14) for the fertilization of eggs deprived of jelly by shaking and subsequent washing.

The same experiment was repeated on July 18, 20 and 21, with identical results: the eggs from which jelly is entirely removed by HCl continue to produce the sperm-agglutinating substance (fertilizin) so long as they live, but their capacity for development after fertilization is much reduced.

In all experiments at least three concentrations of acid were used, and in each experiment it was observed that when the concentration was sufficient to dissolve the jelly there was a good deal of agglutination of the eggs, and in the later washings a great many eggs broke down liberating their pigment. As I have previously shown, broken-down eggs liberate a substance (anti-fertilizin) which neutralizes the sperm agglutinating action of the fertilizin. Therefore, when a sufficient percentage of the eggs are breaking down, the production of sperm-agglutinating substance (fertilizin) by intact eggs may be entirely masked.

I have no intention of disputing Professor Loeb's observations

for *Strongylocentrotus*. But they merely prove either that *Strongylocentrotus* sperm is not so delicate an indicator as *Arbacia* sperm, or that the method employed by Loeb was inadequate to detect small quantities of fertilizin. In *Arbacia* the eggs continue to charge the sea-water with sperm-agglutinating substance after complete removal of the jelly, whether by shaking and repeated washings, or by HCl; and the substance continues to be formed as long as the eggs remain fertilizable and living, no matter how often the eggs are washed. *The eggs of Arbacia secrete the substance as I previously maintained. It is not merely the "dissolved chorion."*

It might possibly be objected to this conclusion that the continued appearance of the agglutinating substance in egg suspensions in sea-water after removal of the chorion indicated merely previous adsorption of the substance of the chorion. But the indefinite continuance of its production is inconsistent with the idea of a mere secondary removal of an adsorbed substance. The idea is also inconsistent with the fact that *Nereis* eggs have no jelly at the time laying, but produce a similar sperm agglutinating substance. In this form the jelly also is secreted by the egg after insemination.

Finally if it can be shown that the jelly of immature eggs is entirely devoid of the sperm agglutinating substance, my position that this substance is a later secretion of the egg is rigorously proved. As noted above I maintained the probability of this view in my previous paper (Study VI). This summer my first experiments were undertaken to investigate this point anew.

Fortunately the season was late, and not a single *Arbacia* was ripe when I began work (June 8). This applied to males as well as females: so it was impossible at first to secure ripe sperm as indicator. I therefore made extracts of immature ovaries to be kept for subsequent testing from three females (1, 2, and 3, June 8). June 11 extracts of ovaries in sea-water were made from females 4, 5, and 6: numbers 4 and 6 contained only ovocytes; No. 5 had a large number of ripe ova in addition. On June 16 extracts 1–6 were tested with *Arbacia* sperm suspension: 1, 2, 3, 4, and 6 were absolutely negative; no agglutination. No. 5 gave a strong agglutination reaction lasting about one minute.

It is highly improbable that the agglutinating substance had been destroyed in five of the six, and retained in the only one (No. 5) of the extracts which was made from ovaries containing some ripe ova. So far as these observations go, the jelly of immature ovocytes is free of agglutinating substance.

Again on June 15 I made extracts from ovaries of three females in two of which ripe ova were practically absent, the third had a few. Tested the same day the two former extracts had no sperm agglutinating properties; the third gave slight agglutination.

The females appeared to mature slightly earlier than the males, so that for these experiments I was forced to use rather thin sperm suspensions (mixed more or less with immature spermatozoa), which were probably not as delicate indicators as one could wish. However the difference between the ovaries containing ripe ova and those without was perfectly distinct. Later when fully ripe males could be had all ovaries contained ripe ova.

The following observation also tends in the same direction: June 27, 1914—Three females were selected, of which number 1 was the ripest attainable, the eggs flowing freely out of detached ovaries, and very few ovocytes occur; numbers 2 and 3 were the least mature attainable; number 2 had very few detachable ova, mostly late ovocytes with a sprinkling of ripe eggs; number 3 had quite a few detachable ova with a large proportion of ripe eggs. The ovaries of all three were cut up equally, and sea-water added to each to make 10 c.c. When the ova and ovaries had settled they stood at 1.5 c.c. in 1, at 1.3 c.c. in 2, and 1.5 c.c. in 3. After five hours, tests of the agglutinating strength of the supernatant fluids were made with clear fresh sperm.

No. 1 gave a 10-second reaction at 1/800 dilution.

No. 2 gave a 6-second reaction at 1/10 dilution.

No. 3 gave a 7-second reaction at 1/40 dilution.

Thus No. 1 is 80 times the strength of 2 and 20 times the strength of 3. In general the fertilizin production is proportional to the ripeness of the ovaries.

There is not the slightest doubt in my mind about the demonstrative character of these observations. The appearance of agglutinating substance in the jelly of *Arbacia* eggs is secondary,

and takes place probably at the time of breaking down of the germinal vesicle.

Loeb's contention that the agglutinating substance is merely dissolved chorion therefore does not hold for *Arbacia*. With this his argument against my fertilizin theory also falls: "Moreover if it should turn out that the substance which is responsible for the cluster formation is identical with the substance which Lillie calls "fertilizin," which is very likely the case, Lillie's theory becomes untenable, since this substance does not, in all probability, originate from the egg, but from the chorion and since there is, as we have seen, no connection between the presence of this substance and the power of the eggs of being fertilized" (pp. 136–137—Loeb, '14).

In this statement Loeb sums up the essentials of his criticism; since I have shown that "cluster formation" is true agglutination (which Loeb now admits), and that the agglutinating substance (my fertilizin) is not dissolved chorion but a true secretion of the eggs which continues to be produced after the chorion is removed, the entire stated criticism becomes ineffective. There *is* a connection between the presence of this substance and the power of the eggs of being fertilized: the substance can first be demonstrated at the time that the power of being fertilized first arises, viz., after breakdown of the germinal vesicle; it can be demonstrated as long as eggs retain the power of being fertilized, whether the chorion be removed or not, and it disappears absolutely after fertilization, as I showed in my previous paper (study VI, p. 553, 1914).

3. Other Criticisms.

Another objection raised by Loeb is that "the supernatant sea-water of the eggs of *Strongylocentrotus franciscanus* will not induce cluster formation of the sperm of *Strongylocentrotus purpuratus:* yet the latter sperm fertilizes the eggs of *franciscanus*," from which he argues that the fertilizin of *Strongylocentrotus franciscanus* can not be necessary for the fertilization of its eggs. An error in logic is involved here; agglutination of sperm is merely an indicator of the presence of a certain substance, which is none the less present in *franciscanus* even if *purpuratus*

sperm does not reveal it; it *may* nevertheless be activated by *purpuratus* sperm and this is the essential point in the theory.[1] Agglutination of sperm is of no significance except as indicator. As I pointed out in my previous paper, binding of the fertilizin by sperm receptors, *i. e.*, the chemical reaction, is a thing entirely distinct from agglutination; if such binding causes a certain kind of physical surface change of the spermatozoa of suspensions of a certain minimum concentration, they agglutinate; otherwise not. Agglutination is a valuable indicator that enables us to make certain analyses, and that is all. The same principle of fertilization may hold in the entire absence of sperm agglutination.

Another objection in which Loeb supports the possibility of superposing fertilization on parthenogenesis will be dealt with in a separate paper. My contention in this case is that the possibility of such superposition always rests upon incompleteness of the parthenogenetic reaction; if the fertilization reaction be complete, whether by parthenogenesis or insemination, it cannot be repeated. Everybody admits that eggs fertilized by sperm cannot be refertilized; it is a logical impossibility that eggs "fertilized" by parthenogenetic reagents should be refertilized. The problem of the apparent contradiction involved in Loeb's and Herbst's contention of superposition works out in the manner indicated. A study of this problem by one of my students will appear soon.

Loeb cites as a farther difficulty of my fertilizin theory, which he says I have not considered, "that in addition to the membrane forming substance still another, namely a correcting agency, is necessary for causation of the development of the egg." Though

[1] Loeb states (1914, p. 135): "If the phenomenon of cluster formation were inseparably associated with the power of the eggs of being fertilized, we should expect that sperm should only be able to fertilize the eggs of a species if the egg-sea-water of the same species caused the cluster formation of the sperm." I have never maintained that agglutination ("cluster formation") is inseparably associated with the power of the eggs of being fertilized, but merely that a certain substance produced by the egg is a necessary factor in fertilization. In some cases this substance (fertilizin) produces agglutination of the sperm of its own species, and this reaction furnishes an indicator of its amount, when present, or of its absence. In other cases such an indicator is lacking: I do not find that supernatant sea-water of the eggs of the starfish (*Asteria forbesii*), for instance, agglutinates its own sperm; but I have evidence, to be published elsewhere, that the mechanism of fertilization may be explained in the same way as in *Arbacia*.

I cannot accept this statement of the problem, I have nevertheless taken into consideration the fundamental fact, to which Loeb alludes, in the full account of my experiments, which appeared after Loeb's paper was in press. The fundamental fact is simply that the fertilization process in some cases can be divided in two sharply marked stages. This is perhaps most simply and convincingly shown by my own experiment (Lillie, 1911) of removing the spermatozoon from the egg of *Nereis* after it had already induced the cortical changes, with the result that the developmental phenomena came to a standstill before the first cleavage. I cannot agree with Loeb that the second stage involves a factor corrective of an excess action of the factor of the first stage. I think it is probable that we have a progressive process readily capable of resolution into two stages.

In my complete paper (Lillie, 1914) I considered the second phase of fertilization with reference to the new theory, and may refer the reader to the discussion there given (study VI, pp. 582–584). Here it is only necessary to point out that the "fertilizin" theory is at least as well adapted to account for the two stages as the "lysin" theory.

4. Conclusion.

I may be allowed to emphasize the essential features of my theory with some added light thrown by the work of this summer. The fundamental conception is that all agencies initiating development of the egg do so by the same means, viz., activation of an ovogenous substance, which I have termed fertilizin. This conception brings fertilization and parthenogenesis under one conception. I further assumed that such activation in the case of fertilization was caused by union of a constituent substance of the spermatozoon (the sperm receptors) with the fertilizin, and that the activation expressed itself by consequent union of the fertilizin with certain egg substances (the egg receptors). The reaction was thus conceived in terms of the Ehrlich side-chain theory, and was represented diagrammatically accordingly.

That certain chemical combinations form an essential feature of the fertilization reaction cannot be open to doubt. I have not previously taken into account the consideration that the

occurence of such reactions, taking place, as they must, across the egg membrane, is dependent on physical conditions of the membrane, especially its permeability to the substances concerned. In speaking, as I did, of five blocks to the fertilization reaction, I was concerned only with the chemical reactions involved. There may be other blocks of a physical nature. Indeed these were much in evidence in the fertilization of *Asterias*, which I studied in the first part of the summer, and shall report on elsewhere. Another important consideration is that the reaction must also be dependent on environmental conditions such as temperature, ionic constitution of the medium (see Loeb, '14*b*), etc. Blocking of fertilization may also arise from such causes.

Continuing the exposition of the theory; I identified the fertilizin of *Arbacia* with the substance found in the fluid of egg suspensions which causes agglutination of sperm suspensions of the same species. This phenomenon cannot possibly be lacking in significance, for it furnishes direct evidence of a combination of egg and sperm derivatives; the phenomenon itself is not concerned in fertilization, for a single spermatozoon may fertilize an egg. Neither does the absence of such agglutination in other species affect in the least the conclusion that may be drawn from *Arbacia:* because we may have a combination of egg and sperm derivatives without any sperm agglutination. The agglutination is incidental, the combination is the essential thing.

The fertilizin theory in its essential aspects is not dependent on the identification of fertilizin and sperm agglutinating substance. I believe in their identity; but if it were proved, as Loeb has sought unsuccessfully to do, that the agglutinating substance is not essential for fertilization, the fertilizin theory would still not be attacked in its essence. The conception that initiation of development is essentially a phenomenon of activation would still stand in opposition to theories of external agents acting directly by corrosion (cytolysis), or coagulation, or what not. The egg could still be regarded as a self-contained system with no more than the usual environmental relations. It is only from this point of view that the complex phenomena of parthenogenesis and fertilization can be united in a logical whole.

The theory of the identity of fertilizin and sperm agglutinating substance rests upon a considerable body of ascertained facts (see study VI), and it gives us at once a point of attack and a working hypothesis of considerable value. I have been able to show for instance that the origin of the capacity of the egg for being fertilized can be understood on this basis; that the cessation of fertilization capacity can also be so understood; and that the physiological sterility (prevention of polyspermy) of fertilized eggs is readily explained by the neutralization of the fertilizin by a substance (anti-fertilizin) demonstrably present in the egg.

On the other hand the theory does not postulate that the fertilizin of all forms should agglutinate sperm of its own species. There may be many forms in which the union of the sperm receptors with fertilizin does not produce such physical changes of the spermatozoa as to lead to agglutination. In those cases in which agglutination does occur we have a reaction very useful in analysis; but it cannot be too strongly emphasized that the agglutination itself is to be regarded merely as an indicator of the essential reaction.

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THE FERTILIZING POWER OF SPERM DILUTIONS OF ARBACIA

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My previous studies of fertilization led to the conclusion that initiation of development depends upon activation of a substance produced by the egg and located in its cortex. This substance, for which I propounded the name *fertilizin*, possesses two side-chains involved in fertilization, one of which unites with receptors borne by the spermatozoon, the other with the receptors borne by the egg. The latter is the fertilization reaction proper; it was postulated that any agent that may activate the fertilizin, as do the sperm receptors, so as to cause its ovophile side-chain to combine with the egg-receptors may act as a parthenogenetic agent. Fertilization and parthenogenesis are thus brought under one point of view with reference to initiation of development.

Certain data concerning the fertilizin of Arbacia, such as time of origin, its location in the egg, its disappearance after fertilization, etc., are known owing to its property of agglutinating spermatozoa of the same species, which serves as indicator. Some of its chemical and physical properties have also been studied to a certain extent. But up to the present the very existence of the so-called sperm receptors has remained hypothetical. On any theory of fertilization it is necessary to postulate the existence of a spermatic substance that induces development of the egg. But in spite of a considerable number of investigations, the existence of such a substance still remains an hypothesis. The present contribution presents demonstrative evidence, though of a negative sort, concerning this substance.

The phenomena, on which I rely for my conclusion, concern the fertilizing power of sperm suspensions of graded dilutions. On the basis of the usual supposition that a single active spermatozoon may fertilize an egg of its own species, all of the eggs should be fertilized in a series of sperm suspensions of increasing dilutions up to the place in the series in which each egg receives only a single spermatozoon. Beyond this the percentage of eggs fertilized should fall off at a certain rate to a vanishing point.

An approximate realization of this may be obtained if the interval between preparation of the more dilute sperm suspensions and their use in fertilization be made as short as possible. This may be done by the addition of eggs to measured quantities of sea-water, followed by the

addition of a sufficient quantity of an accurately determined sperm suspension stirred in quickly to reach the desired dilution. The final dilution is then made in the presence of eggs, and the age of the final dilution is therefore practically zero with reference to the fertilization reaction.

Under these conditions 100% of the eggs may fertilize up to a dilution of about 1/3000 of a 1% sperm suspension. The curve of fertilizing power of the sperm suspensions measured in terms of the percentage of eggs fertilized then falls off slowly to 1/24,000 of 1% sperm, then rapidly to about 1/300,000%, then slowly again to about 1/90,000,000% where, however, about 1% of fertilization may still take place. Accompanying observations showed that beyond a dilution of about 1/5000 of a 1% sperm suspension only a single spermatozoon can possibly be concerned in the fertilization of each egg.

One obtains exceedingly contrasting results if a series of sperm dilutions in powers of 2 is made, beginning with 1%, by first transferring a certain amount of the 1% suspension to a second crystal of the series and adding an equal amount of sea-water, proceeding similarly from crystal 2 to crystal 3, and so on down the series. In such a case one finds that fertilization runs out absolutely from about a 1/64 dilution of 1% sperm suspension to 1/1024% in different cases. No comment is needed to emphasize the contrast.

As many as 20 to 40 active spermatozoa are found in association with each egg at 1/128%, which may be, however, absolutely ineffective. So that in an experiment running out in the seventh crystal of the series it would appear that a greater number of spermatozoa than this is required to fertilize an egg.

These results suggest at first glance that the order of adding eggs and sperm to the sea-water may be of significance. This is, however, not the case. The repeated handling of the sperm in successive half dilutions is also not the main cause for the result. Thus it would appear that the only real difference between the fertilizing power of the sperm in these cases is a time factor. In the first case the final dilution is made in the presence of the eggs; in the second case 20 to 30 minutes is consumed in the preparation of the sperm dilutions before the eggs are added.

The time factor is the real explanation as will be shown immediately. But at first sight this did not seem a very reasonable explanation for the following reasons: In the first place the time involved has never been considered sufficient to reduce fertilizing power of sperm; and in the

second place the original 1% sperm suspension was shown in several experiments to be capable of fertilizing a high percentage of eggs at 1/30,000 dilution or less, after a longer interval of time. If the sperm suspensions lose their fertilizing power with time, it must be that the significance of time in this respect varies inversely to concentration.

This conclusion was abundantly verified by the following tests: A quantity of sperm suspension of a given concentration is prepared and divided in several equal amounts in a series of crystals; the same quantity of eggs is then added at time intervals to the crystals of the series, and the percentages of fertilization estimated by careful counts. Fourteen different grades of dilution between 1/300 and 1/240,000 of 1% sperm were thus measured. Loss of fertilizing power was shown in all of the suspensions thus tested in less than one hour; and in general the rate of loss increased with each successive dilution. The actual data are to be published elsewhere. Here we may summarize the results in the following table, showing the approximate time required A, for 66% loss of fertilizing power, B, for complete loss, at six different dilutions.

Dilution of sperm	1/1200%	1/3000%	1/6000%	1/30000%	1/60000?	1/120000%
A. 66% loss	32 min.	7 min.	5 min.	3 min.	2-3 min.	1 min.
B. 100% loss	64 min.	24 min.	?	20 min.	20? min.	7 min.

Suspensions of higher concentrations, than those included in the table exhibited a much slower rate of loss, which was measured by another method, showing that from 1/4% down, loss occurs in increasing amount within a period of 100 minutes.

Other possible factors than time influencing the fertilizing power of sperm suspensions are on the whole relatively slight in this series of experiments. There is a certain natural variation in different lots of ova and sperm, which is no doubt responsible for some irregularities in the data. Another factor is that of egg-concentration; but a series of determinations showed that the actual variations due to this cause, occurring in the experiments themselves, are not of significance.

There are two modes of explanation of these results theoretically possible, viz: First, that the loss of fertilizing power is due to loss of motility of the spermatozoa; in the first place this theory does not agree with the observation, that the spermatozoa lose their fertilizing power before they lose their motility; in the second place it renders the increase of rate of loss with dilution incomprehensible because on *a priori* grounds the exact opposite would be expected; in the third place as a result of several lines of work the theory that the fertilization reaction is primarily a function of motility of the spermatozoon has been given up; penetra-

tion of the ovum by the spermatozoon is due to the inception of the fertilization reaction, and not the reverse, as was previously assumed. We cannot therefore accept this theory.

The second theory is that the spermatozoa lose their activating substance, sperm receptors in my terminology, which agrees very well with the demonstrated ineffectiveness in spite of the observed persistent motility. That the rate of loss should increase with dilution is to be expected if we regard the loss of the sperm receptors as a diffusion phenomenon; and, if we regard it as an active process of secretion, we should expect such a result owing to general increase of functional activity under conditions which approach more nearly the normal.

The second mode of explanation, which I have actually adopted, fits in with the necessary postulate that the spermatozoon bears such a substance, and with the fact that the spermatozoon carries out the initial fertilization reaction while it is still intact and external to the ovum. The postulated activating substance of the spermatozoon must be liberated before penetration, and these experiments give us some idea as to the manner of its liberation.

Glaser (*Biol. Bull.* 26, 84–91; 1914) has recently maintained that more than one spermatozoon is necessary for fertilization of the same form which I have studied. His observations may also find their explanation under the same point of view, inasmuch as he was not aware of the significance of the time factor in inseminations with highly dilute sperm.

We thus obtain the following additional point of view with reference to fertilization: the spermatozoon arriving at the egg while still intact liberates an activating substance which initiates the fertilization reaction; as one consequence among others of this reaction the spermatozoon is taken up by the egg, and completes the process of fertilization in its interior. This point of view is consistent, as far as it goes, with my own theory of the fertilization reaction; and it is also perfectly consistent with Loeb's quite different point of view.

My previous experiments had shown that eggs lose a certain substance in sea-water (fertilizin) which is necessary for their fertilization; fertilized eggs no longer produce this substance and are incapable of fertilization. Both eggs and spermatozoa, therefore, contain substances, more or less liable to loss, which are necessary for fertilization. The mechanism of fertilization cannot possibly, therefore, be regarded in the simple manner postulated by Loeb's theory. The existence of parthenogenesis demonstrates the efficacy under given conditions of the egg-substance alone; we must therefore regard the spermatic substance essentially as an activator of the fertilizin of the egg.

There remains of course the problem of identifying the free activator in the medium of the sperm suspension by its only known mode of operation, that of fertilizing the ovum. This problem, over which several investigators have broken their weapons, appears in a somewhat different light as a result of these experiments; and new experiments should therefore be undertaken.

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